THE MEASUREMENT OF GENE MUTATION RATE IN DROSOPHILA, ITS HIGH VARIABILITY, AND ITS DEPENDENCE UPON TEMPERATURE[1]

H. J. MULLER
University of Texas, Austin, Texas

Received October 25, 1927

TABLE OF CONTENTS

	PAGE
THE PROBLEM	
The failure of attempts to produce gene variation	280
The need for a means of measuring mutation rate	281
Lethals as a practicable index of mutation rate	284
Temperature as the condition first to be investigated	285
THE METHOD OF ACCUMULATING MUTANT GENES BY THE AID OF "BALANCED LETHALS," AS AT FIRST APPLIED	
Testing for lethals	286
The increase in number of mutant genes in succeeding generations	288
The prevention of differential survival	289
The synthetic balanced stock	290
The maintenance of separate lines of descent	292
The recognition of antecedent mutations	293
Insuring the unity of the source of tested chromatin in a given group of lines	293
The testing of the balanced lethal lines	295
Conditions of rearing of the lines	299
Results—determination of an autosomal mutation rate, and of a probably significant variation in the time-rate of mutation, associated with the temperature difference	301
LESSONS DRAWN FROM MUTATION EXPERIMENTS ON THE X-CHROMOSOME	
The first attempt to estimate the lethal mutation rate	306
Altenburg's establishment of the first significant figure for mutation rate—its unexpected magnitude	308
Joint confirmation of Altenburg's figure, and the securing of a probably significant effect of temperature on the time-rate of mutation	309
Comparison of results obtained on first and second chromosomes	315
The discovery of significant variation in the mutation rate, of unknown origin	317
Corroboration of the unexplained determinate variation in mutation rate	324
FURTHER MODIFICATIONS AND TESTS OF THE METHOD OF BALANCED LETHALS	
Allowing for genetic and cultural heterogeneity	328
Facilitating the final testing of lethals	330
Simplifying the establishment of the lines	332
Automatic preliminary tests of the lines	335
Propagation of the cultures	336
Further experiments	337
THE LATEST TEMPERATURE EXPERIMENT	
The conduct of the experiment	338
The results and their evaluation—the effectiveness of temperature when the duration of the generation is held constant	341
INTERPRETATION OF THE FINDINGS AND GENERAL CONSIDERATIONS	343
SUMMARY	348
LITERATURE CITED	356

[1] Department of Zoology, UNIVERSITY OF TEXAS, Contribution No. 211.

THE PROBLEM

THE FAILURE OF ATTEMPTS TO "PRODUCE" GENE VARIATION[2]

Biologists are in general agreed that the basic problems of organic evolution are concerned largely with the nature, the causation, and the modes of transmission of heritable variations. Great have been the strides of the last quarter century in our understanding of the last mentioned phenomena—those relating to the *transmission* of variations—owing chiefly to the growth and ramifications of the gene and chromosome theories, and these advances, together with the manifold discovery of "mutations" of these genes, have profoundly affected our viewpoint on the former questions. In accordance with these findings, most geneticists at present conceive of heritable variations (at least, of most of those heritable variations which might be of importance in evolution) as consisting in sudden, rare, discrete changes in individual genes, and the problem of variations thus becomes transferred from the germ plasm as a whole, or even from the organism as a whole, to a much minuter (ultramicroscopic) portion of it. Nevertheless, this being admitted, there is nearly as great a lack as ever of positive knowledge concerning the questions first mentioned—namely, the real nature of heritable variation in that material in which it does occur, and the factors causing, conditioning, or influencing that variation.

Numerous claims, have, to be sure, been made, ranging from assertions concerning the general heritability of the effects of training to such specific theses as that of the induction of a given mutation by means of an antibody. And more such claims are being made, almost daily. It is to be noted, however, that the respectability of such claims is in almost direct proportion to their newness; none of those which has had the opportunity to withstand the test of many years, of critical analysis, and of repeated trial, has succeeded in doing so. For the pilotage of modern genetics is essential to steer clear of the mines of heterozygosis and recombination, of delayed or "maternal" inheritance, of varying differential viability, and of unconscious or even "accidental" selection. Exceptional care is sometimes necesssary also to avoid a systematic repetition, in particular lines of descent, of certain environic modifications which may

[2] The present paper was written in the fall of 1926, just prior to the author's mutation experiments involving X-rays (a preliminary account of which has been given in SCIENCE, July 22, 1927). The discussion in the present paper, therefore, must nowhere be taken as applying to this later work, the results of which deviate widely from those of previous mutation experiments. Nevertheless, it is believed that the points made in the present paper still hold, within the limits thus set.

be caused by contagious disease, by special treatment, or by other conditions. The fluctuating personal equation and means of detection employed in the finding of the variations is another important factor. Further, to analyze the results genetically, when obtained, requires special methods. In addition, an elementary knowledge of the theory of probability is usually prerequisite in order to avoid being misled by the mirage of non-significant numerical differences. Sometimes not all of these dangers have been overlooked, but after a careful survey of all this literature on allegedly induced variation, the present writer has been led to the opinion that in none of the reported cases, not even in the recent ones, have any changes of the genes been demonstrated to have been brought about by treatment,[3] nor does such an effect seem to me to have been made even reasonably probable, in the light of genetic analysis. On the other hand, in a fair number of recent cases (e.g. MANN 1923) adequate technique for avoiding the above common sources of error has been employed, and in these very cases the results of treatment, so far as could be determined, were all admittedly negative. It is in this sense that "positive" knowledge may be said to be lacking.

For all that, gene change most certainly does occur sporadically, having been demonstrated most frequently in the more numerous "untreated" cultures used in breeding experiments primarily concerned with gene distribution,—not to mention the cases of mutated genes that have been found in nature. Do the preceding results mean, then, that mutation is unique among biological processes in being itself outside the reach of modification or control,—that it occupies a position similar to that till recently characteristic of atomic transmutation in physical science, in being purely spontaneous, "from within," and not subject to influences commonly dealt with? Must it be beyond the range of our scientific tools?

THE NEED FOR A MEANS OF MEASURING MUTATION RATE

We need be forced to no such hopeless conclusion as might above seem indicated. The simple reason is that the "rate" of mutation, that is, of readily detectible mutation, is probably so low under ordinary circumstances in most of the organisms dealt with in the experiments hitherto carried out, that a 100 percent, or even a 500 percent effect upon it, due to a given treatment, would, with the methods and number of individuals that have been employed, be very likely to escape detection. For ex-

[3] We may except here the case of variegated corn, in which only a single, especially mutable, genetic locus is concerned. We are also excepting the preliminary experiments of ALTENBURG and the present writer, on temperature, which led up to the present work.

ample, in certain experiments in which mutations have been looked for in *Drosophila melanogaster* (popularly supposed to be so exceptionally mutable), scarcely one mutant has been found among 50,000 flies; a figure of the same order of magnitude would be reached by dividing the several hundred (400 ±) mutations found in the collective work on these flies into the twenty million (more or less) flies of this species that have, all in all, been examined. An experiment comprising 10,000 flies is usually considered respectable, yet it will be seen that such a count might easily fail to reveal a single mutation, even though, owing to a given treatment with, say, radium or alcohol, the usual tendency to mutation had been exceeded a thousand percent! On the other hand, if 2 or 3 mutations had been obtained here, this small figure would be practically meaningless, even if personal equation and the other sources of error could be allowed for. In mammals, much smaller numbers are usually dealt with; here, then, a hundred fold increase of the mutation rate, due to treatment, might well escape discovery (supposing that in them mutations ordinarily occurred and could be detected with the same frequency, per animal counted, as in flies).

Previous experiments, then, not only seem inadequate to prove that environic agents "produce mutations," but they also fail to prove that such agents do not "produce mutations,"—if in the word "production" we may include the idea of the causation of a radical increase of frequency of the process (inasmuch as this would involve the appearance of many mutants that otherwise would have been non-existent). All that the experiments do indicate is that mutations cannot, by means of most of the agents and with the dosages used, be produced *en masse*, in such vast numbers as to exceed the ordinary mutation rate perhaps a hundred fold (10,000 percent).

It may be emphasized again here that no ultimate distinction exists between the idea of "production" as such and the idea of a change in rate of occurrence. If, however, mutation is a physical or a chemical reaction depending on certain disturbances of molecular stability that can occur to an appreciable extent even under "ordinary" conditions, it may be very useful to consider it in the manner above suggested, that is, in terms of its rate, just as we consider many chemical reactions in this way, even though mutation must have a vastly slower rate than most reactions with which the chemist deals. The question as to "what agents will produce mutations" may then be changed to read, "what agents will cause a noteworthy change in mutation rate?"

We must certainly consider as "noteworthy" in the present connection

such a change as a doubling of the rate (100 percent increase), just as this would be considered significant in the case of any other kind of reaction. A change of this magnitude in the rate of mutation might be of significance both in its bearing on the nature of mutation and of the mutating gene material and in relation to the process of evolution. The finding of such a change, morever, might be but an entering wedge. For if, by systematic and precise investigations, agents were discovered that produced increases of the above order of magnitude in the mutation rate, it might be that eventually, by combining them, or applying them in special ways, methods might be arrived at whereby vastly greater effects than those first obtained could be achieved, and so the ideal of the "production of mutations" (*en masse*) might finally be realized.

It will now be evident, however, that a new technique will be desirable for attacking the problem of the variation of the hereditary units from this angle. A method is called for whereby enough mutations can be discovered to yield a figure for mutation rate in a *control* series of individuals, which will be large enough, in absolute numbers, so that, when it is compared with a corresponding figure obtained from the treated series, a difference of about 100 per cent, at least, could be recognized as being a "real" or "significant" difference rather than a mere "chance difference" or "error of sampling." This figure of 100 per cent is tentatively chosen because in some already known processes within the organism, for instance some reactions of "basal metabolism," larger changes in rate than this are not commonly produced, while at the same time changes as large as this should be attainable for most chemical reactions.

The idea of developing such a technique, for bisexual organisms at any rate,[4] does not seem to have suggested itself before the experiments preliminary to the present series were undertaken, for the seeming extraordinary rarity of mutations, even in Drosophila, apparently put them beyond the pale of such quantitative measurement. The task of actually counting mutations in ordinary cultures, in order to compare their frequencies of occurrence there with those under other, contrasting conditions would have seemed almost like that of counting needles in haystacks, to compare their frequencies, or like making graphs to show the rates of occurrence of gold pieces on streets of different types. The

[4] In the case of BAUR's recent experiments with self-fertilized lines of *Antirrhinum* the frequency of detectable mutations appears considerably higher than in other organisms examined; this is doubtless due in part to the fact that selfing makes possible, after just one generation, the manifestation of all recessive genes for which an individual may be heterozygous. On the other hand, the relatively high "mutation" rates reported for *Oenothera* are, as has been abundantly shown, due to gene and chromosome recombinations rather than to real changes in the genes.

objective of most genetic counts, therefore, was the determination of the frequencies of crossing over, of chromosome reassortment, of non-disjunction, and of other phenomena connected with the transmission rather than with the origination of gene variations. Meanwhile, mutations were of course recorded as they arose, but the numbers in which they were found were insignificant in any one given experiment, and still meant little even when many different experiments were totalled, because of the fact that the conditions for their detection varied so greatly from one experiment to another—owing to personal equation, to the differing characters being considered, to the different methods of breeding used, the varying external conditions, diverse stocks, etc.

LETHALS AS A PRACTICABLE INDEX OF MUTATION RATE

It had long been held, however, by the present writer, on the basis of theoretical considerations (MULLER 1917, 1918), that the number of mutations resulting in lethal genes probably greatly exceeds the number resulting in genes that cause visible, readily detectable character changes. If this were true, then, counts of the number of *lethals* arising by mutation might yield figures high enough to make mutation rate capable of quantitative study in the sense previously explained. Differences in mutation rate thus discovered could in all probability be generalized, to apply to visible mutations as well, since there are both theoretical and experimental reasons for believing that most lethals, as a class, differ from other genes only in the more drastic and disastrous end results they happen to produce in embryogeny, and not in their essential nature, or in their mode of origin by mutation.

The correctness of the idea of a relatively high lethal mutation rate was then proved by ALTENBURG and by the present writer, both in separate and in joint work (only a fraction of which has yet been published, and that only in the form of preliminary notes). This work will be referred to in somewhat more detail later; it will suffice here to say that the number of lethals that arise was found to be far in excess of the number of "visible mutants," both in the X- and in the second chromosome of *D. melanogaster*; the frequency of origin of the lethals was so high, in fact, that it immediately seemed evident that a quantitative study of mutation, using the frequency of origin of lethals as the index of mutation rate, was indeed possible. Another advantage of using lethals lay in the fact that "personal equation" was thereby largely eliminated: observers will often disagree or be uncertain regarding the existence of a "visible" mutation, whereas, with lethals, detection is objective and there are relatively far fewer border-line cases (of "semi-lethals").

Even with these advantages, however, the work with lethals was laborious, and further serious difficulties and apparent inconsistencies arose, which will be described. It has been the chief aim of the present writer's work during the past 8 years to develop a technique that will overcome these difficulties, and by the use of it, to obtain decisive counts that would establish the effectiveness, or the non-effectiveness within certain limits, of some important environic influence in modifying the rate of gene mutation. It is believed that this object has at last been definitely achieved, and that the data so obtained in the first place furnish information of theoretical (perhaps ultimately of practical) value, and, in the second place, demonstrate the general usefulness of the method for an unlimited amount of further work on the rate of gene mutation under varying external and internal conditions.

TEMPERATURE AS THE CONDITION FIRST TO BE INVESTIGATED

The environmental condition which was first chosen for the study of its effectiveness in altering the rate of gene mutation was temperature. This was decided upon for a number of reasons. There was, of course, the important practical reason that temperature differences are relatively easily produced and maintained. Another reason was that heat is bound to penetrate the organism of *Drosophila* and there to alter decidedly the rate of nearly every chemical reaction: for example, a rise of 10°C, to which it is quite practicable to subject the flies, causes chemical reactions to rise to between 2 and 3 times their former speed. Hence, if mutation involves a chemical reaction it might well be expected to increase in frequency at least 100 percent, with a rise of 10°C, unless its rate is somehow restricted by that of some physical process, or unless there is involved in addition some chemical process or processes of an antagonistic nature, that are accelerated more than those processes which induce mutation. But if mutation is controlled by some "*entelleche*," or "vital force," or "perfecting principle" (see "rectigradation"), or if its rate depends wholly on some unguessed magnetic, sub-atomic, or other physical force little affected by the kinetic energy of molecules, it could scarcely be expected to respond so strongly to temperature change.

Data on effects of temperature would have another point of contact with other problems inasmuch as the rate of evolution must necessarily be limited, for one thing, by the rate of mutation; in fact, given rigorous selection, with other factors equal, these rates would be proportional to one another. Since great differences in temperature are common in nature the experimental results should therefore inform us concerning the ef-

fectiveness of one possibly important natural factor in the differing rates of evolution of different groups of organisms. Then, too, if the answer was positive (that is, heat found effective), temperature regulation might be used to advantage in some practical breeding work, either for the purpose of stabilization, or of promoting variation. Finally, a positive result with temperature could rather readily be followed up in various ways in subsequent experiments, in further analysis of the phenomena, by application of the temperature influence to different points in the life cycle, to one or the other sex, in varying degrees, etc.

The account of the present series of experiments will at first deal chiefly with the earlier stages of the development of that method of attack, involving "balanced lethals," which later culminated in the final experiment indicating the effect of temperature. The earlier work on this method was largely independent of other work, but, concomitantly with it, as well as slightly before and after it, another method was being used and improved, involving the X-chromosome. The results with the latter finally exerted an important influence in forcing modifications on the method first referred to. The account of the balanced lethal work will therefore be interrupted, after the first experiment involving it has been presented, and the parallel investigations on the X will be reviewed. These will be followed, finally, by a description of the latest balanced lethal work, in which that method in modified form was carried through to an apparently decisive result.

THE METHOD OF ACCUMULATING MUTANT GENES BY THE AID OF "BALANCED LETHALS," AS AT FIRST APPLIED.

TESTING FOR LETHALS

There is one very serious obstacle in the way of using lethal mutations rather than "visible" mutations as an index of the mutation rate: this is the greater difficulty involved in detecting them. To understand this, it must be recalled that the great majority of mutant genes with which we can deal are recessive, and that, when a mutant gene is first inherited, it usually occurs in only a single individual, in heterozygous condition. If the mutant gene is in an autosome, before it can become homozygous and in any way recognizable, F_2 descendants must be obtained (if we are dealing with bisexual organisms) from this single heterozygous individual. These F_2 must be derived by the inbreeding of just those F_1 which received the gene in question; only one fourth of the pairs of F_1, if mating were random, would have both their members of the required composition. In the F_2 from these particular F_1 any "visi-

ble" mutation could then be detected (in the ratio 1:3); if, however, the gene were lethal, the experimenter would become aware of its existence only if it happened to be in the identical chromosome with, and linked to, another heterozygous recessive "visible" gene, that was previously expected to occur in the same F_2 in the 1:3 ratio. The absence of some or all of the expected "visible" recessives (causing a departure from the 1:3 ratio expected) would here point to the existence of the lethal, and further tests might be made to confirm this conclusion. In the case of autosomal genes, then, the number of individuals that can be used in a count of the frequency of mutation is limited to the number that can be bred, in a special way, as *grandparents*, and, even so, when lethal or other than visible mutations are being looked for, only those lethals in particular chromosome regions, in which a preordained genetic arrangement was present, can be found.

The case is different with mutant genes in the X-chromosome, for here the males allow all visible mutant genes which they received to be apparent in them by mere inspection; lethals in the X would kill the males directly, however, and so would escape detection. The lethals could only be found by breeding females containing them; they would then kill half of the sons of these females, resulting in a 2:1 sex ratio (in which certain expected classes of the males would be absent or diminished in numbers, if the female had also been heterozygous for known visible genes in the X-chromosomes). Thus the existence of such lethals could not be recognized until F_1, and the number of individuals available for a "mutation count" will, in the case of sex-linked lethals, be limited to the number of *mothers* whose offspring can be examined, whereas in the case of sex-linked visible mutants the count consists of the total number of *male offspring* examined. In *Drosophila*, for the same expenditure of time and labor, the latter count will ordinarily be 50 or 100 times as great as the former (since one generally obtains 50 to 100 sons from each mother bred), and it will be still more in excess of the grandparental count required in the case of autosomal mutants. So far as ease of detection is concerned, then, sex-linked visible mutant genes present incomparably the most favorable material, sex-linked lethals being very far inferior to them in this respect, autosomal visible mutants still worse and autosomal lethals standing decidedly at the bottom of the list.

Thus we see that, although exceedingly large counts seem called for in the mutation work, the counts to be obtained, when lethals are looked for, are limited to the number of grandmothers, or, in the case of sex-linked lethals, to the number of mothers, bred, instead of consisting of

all the offspring inspected, as they do in most experiments on crossing over, non-disjunction, etc. The rate of counting may thereby be reduced to a value perhaps 1/100, or even 1/1000 of the rate in ordinary experiments. This difficulty might seem largely to nullify the advantage attaching to the relatively high frequency of origin of lethals, unless a method could be developed which would radically increase the number of individuals available for a count of lethal mutations, with a given expenditure of time and labor.

THE INCREASE IN NUMBER OF MUTANT GENES IN SUCCEEDING GENERATIONS

In developing such a method, advantage was taken of the fact that if a given lot of individuals, known to contain no mutant genes at the start, is bred through a series of n generations (that is, to "F_n"), and one of the individuals of this last (n^{th}) generation is then tested for mutant genes (by obtaining F_2 from it in the way above described, if autosomal genes are in question), this test will reveal all mutant genes that arose in any of the preceding n generations, in the chromatin ancestral to that which eventually entered the individual in question and was subjected to the test. The ancestral chromatin amounted to a total equalling that of at least one gamete, in each of the n generations; hence the single test, carried out after n generations, really informs us of the number of mutations which occurred (in the chromosome regions investigated) in n gametes, and it is equivalent to n ordinary tests. In this way the value of the tests, the "count" which a certain number of tests represents, may be multiplied n-fold,—according to the number (n) of generations through which the experimenter finds it feasible to carry his cultures and accumulate his mutant genes before making the tests. It is true that the mere raising of the cultures in each generation requires labor in itself, but this is routine, and very small compared with that which would be necessary in the special crosses and examinations involved in the same number of tests.

If this were all there were to our problem, its solution would be comparatively simple, merely requiring that the cultures be carried through a large number of generations before being tested. This by itself, however, would not give us a true picture of the average frequency of mutation, because the chromatin that survives to be tested, either in nature or in ordinary experimental cultures, is selected material. That is, random breeding, and more especially the close breeding of most small experimental cultures, may result in a mutant gene becoming homozygous

several generations after its origin; if it is a lethal, it will then eliminate itself, and even if it is "visible," but causes a reduction in viability, it will have a greater tendency to become eliminated than the chromatin in unmutated individuals. The individuals existing after n generations, therefore, will come from a selected line of ancestors, in which, on the average, fewer than the usual number of mutations had occurred—how many fewer could not be calculated accurately. Our next task, therefore, must be to stop the process of "natural selection" (as it occurs in ordinary material).

THE PREVENTION OF DIFFERENTIAL SURVIVAL

This end was accomplished by the expedient of artificially constructing a stock containing "balanced lethals." In such a stock, both homologous chromosomes of a certain pair are, in all individuals alike, equally and maximally handicapped for the selection process to start with, by having a lethal gene already inserted into them. Thus, if another lethal arises in these chromosomes later, by mutation, this new lethal cannot put the chromosomes to any furthur disadvantage in selection.

The lethals intentionally inserted into the two given homologous chromosomes of each individual are different ones, lying at different loci, otherwise the individual would be a homozygous lethal and could not live; since, however, the two lethals are in different loci, each of the two chromosomes in question in the individual contains, at the locus where the other chromosome contains a lethal, a normal gene which is dominant to that lethal and so saves the individual's life. Neither of these lethal-bearing chromosomes is at a selectional disadvantage, *as compared with the other*, since both are lethal and neither could ever survive in homozygous condition. There are, however, no normal chromosomes to compete with them so long as individuals of such a stock are bred together exclusively. Such interbreeding of any two individuals of the doubly heterozygous stock must result in the formation of zygotes in the ratio:1 homozygous for one of the lethals (this will die) :2 heterozygous like the parents (these will live) :1 homozygous for the other lethal (this too dies). The heterozygous balanced lethal condition hence automatically perpetuates itself. To be sure, if there were crossing over between the lethals some crossover homologous chromosomes, having neither lethal or both together, would be formed, and this would upset the "balance," but crossingover can be prevented or hindered by the employment of special factors, or rather, genetic conditions, hitherto called "C factors," in one of the chromosomes, which interfere with its exchanging parts with its homologue.

This balanced lethal condition will prevent natural selection from tending to eliminate chromosomes (of the pair in question) in which further recessive mutations—lethal or merely deleterious—occur, because these chromosomes never have the opportunity to appear homozygously anyway, on account of the lethals in them from the start, and so the new mutant gene can never exert its deleterious influence. A mutated chromosome will, in other words, have as good a chance of surviving as will its non-mutated sister chromosome, present in a sister individual, since both alike can only survive if heterozygous, and then can survive equally well. The chromosome, being already *maximally* "handicapped" by the original lethal in it, cannot have its survival value decreased any further by acquiring another lethal, and "natural selection" is thus prevented.

The idea of making use of balanced lethals for this purpose readily suggested itself, as a result of the writer's previous investigation of the case of "beaded wings,"—in which he found that such a condition had arisen "automatically," in the course of a selection for beaded that occurred before the stock came into his hands.

THE SYNTHETIC BALANCED STOCK

The writer chose the second chromosome of *Drosophila melanogaster* for the artificial balanced lethal arrangement because, at the time the work was started (1918) this was the only chromosome in which "*C* factors" were known that would prevent crossing over throughout most of the extent of a chromosome. The individuals of the stock first used were provided, in one of the members of their "second" pair of chromosomes, with the "*C* factors" termed "$C_{II\ L}$" and "$C_{II\ R}$" (found by STURTEVANT), the first of which prevents, when heterozygous, practically all crossing over in the "left" half, and the second, nearly all crossing over in the "right" half of this pair; in the region in the middle there is, however, a small amount of crossing over. With each of these "*C* factors" there was also associated a lethal factor, practically inseparably linked to it ($l_{II\ L}$ and $l_{II\ R}$, respectively): these furnished the initial lethal effect for one of the chromosomes. The recessive mutant genes for "plexus" venation (p_x) and for "speck" on thorax (s_p) also happened to lie in this same chromosome, at the right end. As they did no harm, and would have been difficult to remove on account of the hindrance to crossing over, they were allowed to remain.

The homologous chromosome was furnished with the gene for "star eye" (*S*) at the "left" end, as its lethal; this has a dominant visible effect upon the eye but the lethal effect is recessive. In addition, since

there was some crossing over in the middle, this chromosome was provided, at its "right" end, with the gene for "morula eye" (m_r), which, though not lethal, causes sterility in the female and hence is somewhat similar to a lethal in propagative value. Between these two terminal genes star and morula, five recessive genes for visible characters, in loci scattered rather far apart, were included: these were the genes for "vortex" on thorax (T^v), "black" body (b), "purple" eye (p_r), "curved" wing (c), and "arc" wing (a_r). They were inserted into the chromosome so that, when the tests for lethals were finally made—in stock in which crossing over was again allowed, to get rid of the lethal, star, as will be explained—the existence of any new lethal that had arisen by mutation in this chromosome could easily be recognized, by reason of the non-manifestation, in F_2 of the test crosses, of the genes to which this lethal was most closely linked. The locus of the lethal could be approximately determined at the same time by noting the relative frequencies of appearance of the recessive characters dependent on these different loci. Unfortunately the gene for vortex, which was the only mutant gene then available in the chromosome region in which it lies, requires a recessive intensifier ($v_{x\,\mathrm{III}}$), located in the third chromosome, to allow it to show regularly when homozygous; the stock had therefore to be supplied with this intensifier.

As finally made up, then, the balanced lethal stock first used had the following composition, representing the genes in each of the homologous chromosomes concerned on separate lines:

$$\frac{S \quad T^v \qquad b\ p_r\ c \qquad a_r \qquad m_r}{l_{\mathrm{II\,L}} \quad C_{\mathrm{II\,L}} \qquad C_{\mathrm{II\,R}} \quad l_{\mathrm{II\,R}} \quad p_x \quad s_p} \qquad \frac{v_{x\,\mathrm{III}}}{v_{x\,\mathrm{III}}}$$

It will be realized that the construction of this stock, started in the fall of 1918, required nearly an academic year, since few of the adjacent genes of the upper chromosome were already connected together, and the addition of $v_{x\,\mathrm{III}}$ also presented complications. The work with the latter gene had to be carried on "in the dark," as it were, inasmuch as neither of the genes, T^v or $v_{x\,\mathrm{III}}$, can manifest themselves unless both of them are homozygous simultaneously, and, in the stock to be made up, T^v had to be heterozygous.

Later in the year, the easily recognizable recessive character "dumpy" wings arose in the Drosophila laboratory as a mutation, and the present writer found, in analyzing it, that it was allelomorphic to, that is, in the same locus as, vortex (T^v); its gene may be designated as T^d. There was time to insert it in place of the less readily detectable gene for vortex in

some of the cultures of the experiment; and in these cultures $v_{x\ III}$ was not added, since dumpy requires no intensifier for its manifestation; otherwise, however, the stock containing dumpy was constructed like the other stock.

THE MAINTENANCE OF SEPARATE LINES OF DESCENT

It might be imagined that all that was now necessary was to raise several large mass cultures of these balanced stocks, carry them through a considerable number of generations ("*n*") and then apply the breeding tests for lethal mutations to a large number of individuals from each culture. Such a technique would, however, be inadequate, for the different tested individuals from a single culture might be more or less closely related; we should have no way of knowing in what generation the lines of ancestry of their tested chromosomes converged, and consequently we could not tell how many ancestral chromosomes the tests really applied to. In other words, common ancestors should not be reckoned more than once in the accounting, but in mass cultures it cannot be determined during how many generations of the ancestral cultures two given individuals have had a common ancestor. There is little or nothing to be gained, therefore, in testing more than one individual from each culture, even though the latter is a large mass culture.

It follows from the above that in order to be able eventually to test a large number of individuals all of which have lines of descent that are known to have remained separate since the beginning of the experiment, it is necessary to maintain in each generation an equally large number of separate cultures, or "lines" of descent, to carry each of these lines through the series of n generations separately, without allowing inter-breeding between it and the other lines, and finally to take just one sample individual from each line, for the crosses which are to give the test for mutant genes. The separate cultures, with this method of breeding, may be reared in small containers (vials), only large enough to prevent too great risk of the lines becoming extinct.

If, now, we have l separate lines which have been bred, on the average, through n generations, and we then test one individual from each line, our l tests will, according to the mode of reckoning previously explained, inform us of the number of mutations that occurred (in the chromosome loci tested) in ln individuals. The size of the "mutation count," then, is the product of the number of lines tested by the number of generations.

THE RECOGNITION OF ANTECEDENT MUTATIONS

From our total number of mutant genes, found among these *ln* individuals, it is of course necessary to exclude any lethals or other mutant genes that arose in the germ plasm before the specified *n* generations of breeding began. This means either that we must, at the start of the *n* generations of breeding, make preliminary tests of that portion of the chromatin of each line which will be ancestral to the chromatin finally tested in that line (this would double the total number of tests necessary) or that we must devise some method of distinguishing, in our final tests, between those mutant genes that were originally present, and those that arose in the course of the *n* generations of breeding. The latter object can be achieved by having the lines related in groups, in some known definite way, at the start of the experiment.

The "sister lines" of a given group, having each of their chromosomes (of the type to be tested) immediately derived from a common ancestral chromosome in the common ancestor of the group, will share any lethals (or other mutant genes) which may unintentionally have been present in that common ancestral chromosome; if such were present, then, the sister lines will contain identical lethals. The identity of these lethals, once they have been discovered in the final tests, can easily be established by crossing them together and determining whether the combination effect is lethal also. On the other hand, those lethals which were not present at the start, but have arisen independently, after the lines of a group were split off from their common ancestor and the experiment proper began, should not coexist as identical lethals in all the lines of the group.[4] Such lethals only will be given a place in our "mutation count."

INSURING THE UNITY OF THE SOURCE OF TESTED CHROMATIN IN A GIVEN GROUP OF LINES

In making the final tests, we must be certain that the chromatin tested in a given line is really descended from the supposed ancestral chromatin

[4] It is true that BAUR'S work on "premutation," and the gene-element conception, indicate that identical mutations may really be expected more often in related than in non-related lines, but in the actual data from our first mutation experiment with the balanced lethal stock, where there were usually 4 to 8 lines in a group, a given mutant gene appeared either in all the most closely related lines of the group or in only one line, never in part of the lines in such a way as "parallel mutation" would have produced. Hence such a tendency was not strong enough, in this work, to have caused an appreciable proportion of the mutations that occurred after the splitting of the lines to be reckoned as having occurred beforehand, and the above method remained valid here. But even if there were a strong tendency to "parallel mutations" in related lines ("premutation") it would still be valid to exclude these, when they could be recognized, and compare the residual numbers of "independent mutations" in different series of lines to determine the effect of the given agencies upon the rate of occurrence of these mutations.

which was either directly tested, by preliminary tests, or, as above stated, tested later by means of the final tests of the other "sister" lines of the group. This condition requires all the flies of a culture to have their chromatin (of the type for testing) derived from a single ancestral source—a result which could not be achieved by using a pair of flies of similar composition to start the group of lines, for in that case some of the flies of later generations might derive the chromatin in question from the original male, and others from the original female. It is accordingly necessary to know that a single member of the original pair, which member may be called the "source" individual, has supplied the chromatin (of the type in question) to the later generations of the sister lines of a group. Furthermore, since this source individual must be diploid, we must likewise know that all the chromatin in question was derived from the same genetic half of that source individual.

These purposes can be carried out by using as the source a single heterozygous male (preferable to a female for this purpose since in the male there can be no crossing over whatever), in which the chromosome in question is distinguished from its homologue by a "visible" gene or "identifying factor" (ALTENBURG and MULLER 1920) that will show in the next generation. This male is crossed to a female which does not contain a chromosome like the "source chromosome" just referred to, but contains the chromosome which is to be the partner of the former in the balanced stock. This "partner chromosome," when in this female, must exist in heterozygous condition, since it is necessary for it to contain a "balancing" lethal; the other homologous chromosome of the female, which is not to be used later, must then be marked off from the "partner chromosome" by another "identifying factor." When the cross between such a male and female is made, the offspring of the required balanced-lethal type can be distinguished from the rest by their "identifying factors," and used to form the start of the sister lines; they all necessarily derive their chromatin of the type to be tested from an identical source.

In the first mutation experiment involving the balanced lethal lines, the groups were started by using pairs (or one male and several females per culture) of the following composition:

$$\frac{S T^{v} b \quad p_{r} c \quad a_{r} \quad m_{r}}{l_{\text{II L}} C_{\text{II L}} C_{\text{II R}} l_{\text{II R}} p_{x} s_{p}} \; \frac{v_{x\text{III}}}{v_{x\text{III}}} \; ♂ \times \frac{l_{\text{II L}} C_{\text{II L}} C_{\text{II R}} l_{\text{II R}} p_{x} s_{p}}{b \; p_{r} \quad c \quad p_{x} s_{p}} \; \frac{v_{x\text{III}}}{v_{x\text{III}}} \; ♀$$

Here the genes S, p_x, and s_p were the "identifying factors" in the source male, and b, p_r, and c were the "identifying factors" in the females. Of the 4 possible zygote combinations formed in this cross, it will be seen that only

one class of offspring appeared star, but otherwise normal; these were the offspring having the required balanced lethal composition, and, in any one culture, all the offspring of this type derived their chromatin of the kind to be tested from one source, namely, from one genetic half of the one male parent. Such offspring were picked out, and bred together in mass culture—one mass culture from the offspring of each original male—to form the first "sister" line of the group of lines from that male.

The second "sister" line of a given group was formed, in each case, by taking a second single male from among these offspring, that is, a brother of the mass culture used to form the first sister line, and crossing it to females like those first used

$$\left(\text{namely, } \frac{l_{\mathrm{IIL}}\, C_{\mathrm{IIL}}\, C_{\mathrm{IIR}}\, l_{\mathrm{IIR}}\; p_x s_p}{b \quad p_r \quad c \qquad p_x s_p}\; \frac{v_{x\mathrm{III}}}{v_{x\mathrm{III}}}\right).$$

As the second male had a composition like that of the source male of the group it in turn produced offspring from which, by selection of the star, but otherwise normal appearing, flies, a second mass culture (the second sister line) could be started, and from which likewise a single male could again be isolated for crossing as before, in order to establish the third and eventually still further lines. So the process was continued until, in some of the groups of the series containing vortex, 9 "sister" lines had been established. It will be noted that the lines thus termed "sisters" are not sisters in the ordinary sense of the term, but really stand in direct descent, one from the other. This particular type of relationship allowed of the test of the frequency of "parallel" mutations, referred to in the previous footnote.

Although preliminary testing of the lines was avoided by having them grouped in this way, considerable labor was nevertheless involved in making all the crosses and selections (especially the selections of virgin flies) necessary in getting the lines started. The establishment of the lines of the "vortex series" was begun in May and continued until November, 1919, nine generations later. At that time the stock containing dumpy in place of vortex was ready, and the establishment of these lines was begun, by means of the same kind of procedure, and carried on through six successive generations. In all, 94 vortex-containing and 249 dumpy-containing lines were started.

THE TESTING OF THE BALANCED LINES

To complete our preliminary account of the balanced lethal method, there now remains to be described the procedure necessary for making

the final test of the sample individual from each line. In order to become aware of what recessive lethals or other mutant genes the chromatin in question, of a given fly, contains, it is of course necessary to give that particular chromatin an opportunity to become homozygous. This cannot be accomplished by simply mating together two individuals of the same culture and thus allowing this chromatin to come into combination with the corresponding chromatin of the other individual, because, firstly, that corresponding chromatin may not be identical with the first and so may not contain the same mutant genes (owing to mutation having occurred since the two branched off from their common ancestral chromatin), and because, secondly, the lethals that had been intentionally inserted to preserve the "balance" would prevent the homozygotes from appearing anyway. A single "sample fly" must therefore be taken from each line of the experiment (in the "F_n" generation), and outcrossed with a fly from a different stock, that does not contain a chromosome similar to that being tested. Those F_1 or rather, F_{n+1}, flies from this cross which show, by their "identifying characters," that they have received from their heterozygous parent of the experimental line the chromatin which is to be tested, are then crossed together, to allow an opportunity for F_{n+2} individuals homozygous for (portions of) this chromatin to be formed.

In this latter cross, there must be a means of preventing the original "balancing" lethal—in our case, star—from killing all the F_{n+2} flies that might be homozygous for any of the chromatin in question; otherwise the effect of any new lethal or other new mutant gene in this chromatin would be obliterated. Such obscuring action of the balancing lethal will be prevented naturally, in some of the F_{n+2} flies, if crossing over between this lethal and the rest of the chromosome is allowed in F_{n+1}, for some of the crossover F_{n+2} flies will then receive from one of their parents (the F_{n+1} female) part of the chromosome to be tested, without this lethal being attached to it, and from their other parent (the F_{n+1} male) the entire chromosome to be tested. The original lethal, being only heterozygous in such an F_{n+2} fly, will then fail to kill it, but the fly will be homozygous for part of the rest of the chromatin to be tested. Which part is thus homozygous will be revealed by the manifestation of the recessive visible genes previously placed there (in our present case, T^v, b, p_r, c, a_r, and m_r). If, now, the homozygous chromatin of the given region contains a visible mutant gene that arose in the course of the experiment, this gene may be seen to produce its characteristic effect in the fly (barring interference between its manifestation and that of the previously provided visible mutant genes), but if this homozygous chro-

matin contains a newly arisen lethal the fly containing it will die even though it is not homozygous for the original lethal (star). Since all such flies in the given culture will be killed, the existence of this lethal can therefore be inferred by the very absence of a certain class or classes of crossover flies that would otherwise be expected to appear, and the locus of the lethal can be estimated by noting just which classes of crossover flies are absent, and to what extent other classes are reduced in numbers.

In applying this method in the present instance a single fly—a male, to avoid all crossing over here—from the "*n*th" generation of each culture, was mated to a female not containing $C_{II\ L}$. The latter is the "factor" which, when heterozygous, prevents crossing over in the left half of the chromosome. As this half is the region containing the locus of the original lethal, star, which it is intended now to get rid of, by crossing over, it will be seen that in the offspring (F_{n+1}) of this female the desired crossing over can now take place. The female was, however, provided with the combination $C_{II\ R}\ l_{II\ R}\ p_x\ s_p$, in order that the determination of new lethals in the right half of the chromosome might not be obscured by too much crossing over. She was only heterozygous for this combination, since it is itself lethal, and she was provided in her homologous second chromosome with the "identifying factors" arc and morula ($a_r\ m_r$). In addition, she had been made up to contain, in homozygous condition, the vortex intensifier, $v_{x\ III}$. Her composition was therefore as follows:

$$\frac{C_{IIR}\ l_{IIR}\ p_x\ s_p}{a_r\ m_r} \quad \frac{v_{xIII}}{v_{xIII}}$$

It will be seen that when such a female is crossed to a male from one of the vortex-containing experimental lines, one quarter of the (F_{n+1}) zygotes formed will have the desired composition, namely:

$$\frac{S\ \ T^v\ \ b\ \ p_r\ \ c\ \ a_r\ \ m_r}{C_{IIR}\ l_{IIR}\ \ p_x\ \ s_p} \quad \frac{v_{xIII}}{v_{xIII}}$$

These will be distinguishable from the others by having star eyes and being otherwise normal. They contain the test-chromosome, but not a chromosome that prevents crossing over between star and the other genes. In the case of the dumpy-containing cultures, a cross to the same kind of female was made, although here the presence of $v_{x\ III}$ was not necessary.

The star F_{n+1} males and females from each culture were then crossed *inter se*, virgin females being used, and the F_{n+2} of each cross were ex-

amined to see whether all parts of the "test-chromosome," except the star-containing region to the left of T^v or T^d, manifested themselves in some flies or other, in the homozygous condition. That is, the observer had to make sure that there were at least a few flies, in the culture, that combined the characters of T^v (or T^d) and b (these were homozygous for the region T^v-b), some that combined b and p_r, some that combined p_r and c, and some c, a_r, and m_r (which practically never separated because of the $C_{II\ R}$). It was also noted whether or not any of these combinations that appeared showed any new visible mutant characters.

The above determination required the obtaining of a considerable number of flies in the F_{n+2} cultures, since only about 8 percent of cross-overs occur between the S and the T locus, and of these 8 percent only one fourth would form recessive homozygotes, and they would have a relatively low viability. There were many cultures, therefore, in which flies of the T^v-b combination failed to appear, even in cases in which there was no lethal in this region, just because of the relatively large "error of sampling" that applies to such small expected numbers. All the cultures that gave such results were therefore regarded as "doubtful cases" at first, that had to be followed up, in later generations, to make sure whether or not a lethal were really present. This following-up process was especially cumbersome, as flies of the requisite composition for continuing the study could not be recognized with certainty in the F_{n+2} population. The same difficulty applied in each generation in which the lethal and the "doubtful" cultures were continued for more exact locus determination. In the locus determinations of lethals that proved to be in the right half of the chromosome outcrosses had later to be made to remove the chromosome containing $C_{II\ R}$, since the latter, by preventing crossing over in this region, made the mapping of the lethal impossible.

Finally, after rather extended special crosses, the detailing of which would take us too far afield, balanced stocks were again synthesized, in the case of all "test-chromosomes" containing a newly discovered lethal. These stocks would maintain this lethal automatically in heterozygous condition, without crossing over, and without further selection being necessary. In these stocks, the original lethal, star, had been removed by previous crossing over, and so outcrosses of these made a more unhampered study of the new lethal possible. Thus, when these stocks were crossed with one another, recessives showing the combination T^v (or T^d) b p_r c a_r m_r could appear in one fourth of all their F_1 offspring—*except* where the lethals in the two stocks happened to be in identical

loci. All possible crosses of this type, between stocks having lethals located in the same general chromosome region, were then made, in order to find out just which lethals did lie in identical loci (that is, were "allelomorphs").

It will be noted that, in the above work, only the $S\ T^{v}$ (or T^{d}) $b\ p_r\ c\ a_r\ m_r$ chromosome of the balanced lines was investigated for new mutant genes. The other chromosome—containing $l_{II\ L}\ C_{II\ L}\ C_{II\ R}\ l_{II\ R}\ p_x\ s_p$—could not be subjected to testing, because the "C factors" here prevented this chromosome from getting rid of the "balancing" lethals $l_{II\ L}$ and $l_{II\ R}$, that were present in it from the beginning, and so the existence of no new lethal recessive mutant genes in that chromosome could be ascertained.

CONDITIONS OF REARING OF THE LINES IN THE FIRST EXPERIMENT UTILIZING BALANCED LETHALS

The 343 lines, which were to be subjected to the "n" generations of breeding, followed by testing of the sort above described, were intended mainly to provide a preliminary series of "control" results that would give an estimate of the frequency of mutation in the second chromosome under certain definite or "standard" conditions, which could readily be reproduced, and which would *a priori* be likely to give a relatively high mutation rate. It was planned later to run other experiments in which the lines were to be bred under other conditions, after the "control" results had been obtained, and then to compare the special with the control results in order to determine the effect on mutation rate of the special methods of breeding or treatment later used. For this reason most of the lines in the preliminary experiments were carried on in one standardized fashion, which involved keeping them in 4 by 1 inch vials, in an incubator at a temperature of 26.5°C. It should be stated, however, that previous to November 7, 1919, when only the vortex series was being established or propagated, the cultures were kept at room temperature. After that, the vortex lines and most of the dumpy lines were placed in the incubator, where they were able to develop at approximately the maximum rate. In the incubator the temperature seldom varied more than 1°C, except for short periods in the summer of 1920, when the room temperature (in COLUMBIA UNIVERSITY, New York City) became higher. The food used consisted of freshly prepared 1 percent agar in 5 parts water, 2 parts mashed banana, and 1 part karo syrup, sprayed with a suspension of yeast, after gelling, and sprinkled with white confetti.

One week after the parents were placed in a culture at 26.5°C, they were thrown out, and two week later the offspring (a sufficient number of which had then hatched) were tansferred directly, *without etherization*, to a fresh culture vial, in which the above cycle was repeated. These fortnightly transfers were carried on for approximately a year (more in the case of lines that had been established earlier, less in those established later). This work of mere propagation was for the most part performed by a supervised assistant, although of course all the crosses necessary for synthesizing the stocks, establishing the lines, testing them, and investigating the lethals, were carried out by the writer. The fact that very few lines at the end showed the effects of contamination indicated that the work of propagation had been carried on with due care, for the germ plasm of a fly without lethals, that had entered a culture, would tend rapidly to upset the special mechanism of "balancing," and to supplant the lethal-bearing germ plasm, by a process of natural selection.

The opportunity could not be resisted, however, of carrying on a minority of the cultures under a different condition, in order that some idea might be obtained of the controllability of the mutation rate in the second chromosome, before too many years elapsed. Temperature was the condition which it was chosen to vary, for the reasons previously given, and because some suggestive results had already been obtained with it on the X-chromosome in the summer of 1919, as will be explained. As there were not facilities for securing a constant temperature much below 26.5°C, and as *Drosophila* does not withstand much higher temperature, the writer contented himself with making the difference in this experiment one of sign rather than of fixed quantity. The vials containing the "cooler" lines were hence kept at the room temperature of COLUMBIA UNIVERSITY, in the cold weather, and in the warm weather they were kept in dishes covered with wet cloths connected with trays of shallow water; over these wet dishes an electric fan was usually kept playing. In this way, the cooler vials were kept about 8°C cooler than the others, on the average, throughout the year. This difference in temperature was reflected in a slower rate of development, and in the case of these vials it was feasible to make transfers, on the average, only once a month. One hundred and thirteen of the lines, all belonging to the dumpy-containing stock, were reared under the cooler conditions. The cooling treatment was started November 7, 1919, when the first lines of this series were established, at the same time as the incubator treatment of the other lines (94 vortex and 136 dumpy) was instituted.

Before the differential temperature treatment in this first balanced lethal experiment had been begun, and during the time that the vortex-containing lines were being synthesized, established and propagated, certain mutation experiments on the X-chromosome were being initiated, in which it was not possible to use the method of balanced lethals. The results of these were obtained prior to those from the fore-going experiments, and, as has been mentioned, they had an important bearing upon the conduct of the later experiments on balanced lethal lines. However, to preserve the consecutiveness of the present account, the results of the balanced lethal experiment which we have been describing will be presented before those of the X-chromosome work.

RESULTS—DETERMINATION OF AN AUTOSOMAL MUTATION RATE, AND OF A SIGNIFICANT VARIATION IN THE TIME-RATE OF MUTATION ASSOCIATED WITH THE TEMPERATURE DIFFERENCE

The balanced lethal lines, started at COLUMBIA UNIVERSITY and at WOODS HOLE in the spring, summer, and fall of 1919, in the manner above described, were tested, by the methods explained above (pp. 296-9) in the fall and winter of 1920-21, after they had been moved to the UNIVERSITY OF TEXAS. When the total number of "chromosome-generations" (the product $l\ n$) in each series had been determined (in practice, by adding the numbers of generations in the individual lines, since not all lines were kept for the same length of time), it was found that in the vortex-containing group (all kept at 26.5°C for the last 11 months, comprising 7/8 of the generations) there was a total of 1918 tested individuals, or "chromosome-generations" (obtained by testing the 73 lines surviving out of the original 94); in the dumpy-containing "warmer" group (also at 26.5°C, throughout the experiment) there were 2180 chromosome-generations" (from the 106 lines surviving out of the initial 136), and in the dumpy-containing "cooler series" 726 chromosome generations (from the 71 lines left out of 113).

In the vortex lot there were 8 new lethals and in the dumpy-containing lot that was also kept at the warmer temperature there were 16. No visible mutations were detected. In other to determine whether the difference between the mutation rates in these two lots is "significant" we may apply the usual formula for the probable error of a difference between small proportions: that is, $0.6745\sqrt{\frac{P}{n_1\,n_2}}$. P in this case is the absolute number of mutations in the two lots taken together, namely

24, n_1 is the total count of tested chromosome-generations in one of the lots, namely 1918, and n_2 that in the other lot, namely 2180. Substituting these numbers in the formula, we find that the difference in rate here is only twice its own probable error, and hence practically without significance by itself (chance 1 in 5),—despite the fact that the stocks were not genetically identical. It may therefore be legitimate, for some purposes of comparison at least, to average the two counts together; we then obtain a mutation rate of 1 lethal in about 170 chromosome-generations, or 0.58 percent, for the second chromosome, in these combined warmer lots.

It has been explained that this was not the first figure ever obtained for mutuation rate in any chromosome, as some of the results of the X-chromosome work, which will be reviewed later, were obtained previously, but it was the first figure obtained for an autosome. As will be seen, it was of the same general "order of magnitude" as most of the figures obtained with the X, although, on account of the great variations found in the latter, exact comparisons with them would be of little value. This "order of magnitude" of the lethal mutation rate is higher than that which had been expected. It implies a rather rapid deterioration of the germ plasm when protected against natural selection, for 1 lethal per 170 chromosome-generations, when the generations occupy two weeks, means that, on the average, there will be one lethal to each unselected second chromosome after the passage of six and a half years, two lethals per chromosome after thirteen years, etc. In terms of individual genes, of course, the rate is much slower. If we consider each long autosome as containing at the very least 600 genes (a revised figure based on calculations given by the writer in 1926), then each of the contained genes will, on the average, give a lethal or otherwise detectable mutation not oftener than every 3,900 years, provided this rate of mutation continues.

In the much smaller "cooler series" only 2 lethals were found—a rate of only 1 in 363, or 0.27 percent. This count in the cooler series is so small that the difference in rate of mutation, per chromosome-generation, between all the warmer and the cooler lines turns out to be only 1.6 times its probable error, when we apply the formula given above. This result, then, "lacks significance," if taken by itself, as the chance of obtaining as great a difference, in either direction, if the rates were really equal, is as high as 1 in 3.3. It will be noted, however, that the difference is in the same direction as in the temperature experiment on the X-chromosome, and in the same direction as most heat effects; the chance

of obtaining such a difference in this direction is of course only half as great.

It has been stated that the lines kept at the lower temperature took twice as long to develop as the others,—the length of time necessary for their cultures to produce a sufficient number of flies for transferring being four weeks, as contrasted with two weeks in the other series. If mutation occurs at a fixed time-rate, regardless both of the temperature (and the consequent speed of metabolism), and also of the stage in the life cycle, and the cell cycle, in which the genes exist, the cooler lines which had passed through 10 generations should tend to produce the same number of lethals as an equal number of warmer lines which had passed through 20 generations in the same length of time. In that case, it would be more legitimate to measure the mutation rate in terms of units that may be called "chromosome-months," rather than in "chromosome-generations." When this method of measurement is employed, the tests on the cooler lines are found to make twice as respectable a showing as before, in total "units" counted, relatively to the tests on the warmer lines. In accordance with this relation, the difference between the mutation rates of the two series, per "chromosome-month," is much greater than that per "chromosome-generation." The time-rate of mutation, in this sense, is 1.17 percent in the warmer cultures and 0.31 percent in the cooler and the difference, 0.86 percent, is subject to a probable error due to random sampling of 0.3 percent. Thus the difference in the time-rates is 2.9 times its own probable error—a "chance" of only 1 in 18.5, regardless of direction, or 1 in 37, if we consider the direction of the change as specified. If we confine our reckoning to the "dumpy"-containing cultures exclusively, we find that the difference in time-rate of mutation between the warmer and the cooler sets is 3.7 times its probable error, a result that would ordinarily be regarded as convincingly positive.

The data therefore seem to yield fairly good evidence that the assumptions on which the latter calculations were based are, one or more of them, incorrect: that is, we are led to conclude that probably the time-rate of mutation is not independent both of temperature, of the speed of "vital activities," and of the stage in the life (or cell) cycle, and that therefore temperature, whether or not it effects mutation frequency directly, can at least effect it indirectly, or through its influence on some of these phenomena. It may perhaps be claimed here that such an effect might have been taken for granted before-hand, but it is by making such assumptions gratuitously that biology progresses over-slowly.

Knowing as we do nothing about the mechanism of mutation, we could not be sure in advance that its speed is limited by that of a chemical reaction and that it hence is highly responsive to temperature changes; still less could we presume to say that the reactions of ordinary "metabolism" are the causative agents in it. Neither have we, until the past year at least, had any valid evidence (except in the seemingly special case of variegation in corn), indicating that mutation, in the sense of alteration of the gene, occurs preferentially at any particular stage in the germ track cycle, though there has been a little evidence contrary to this idea (MULLER 1920). Unless such a relation existed, the mere breeding of individuals at an earlier age would not result in the occurrence of more mutations after a given long period of time, for the mutations would simply have gone on occurring with the same frequency as otherwise, regardless of the fact that the maturation period, etc., had been passed through oftener. It is therefore of importance to measure the frequency of mutation, under various conditions, not only in terms of its rate per generation, but also per unit of time. It is in terms of time units, also, that the effect of temperature on chemical reactions in general is usually measured, and so a comparison with the latter may best be made in these terms.

Unfortunately, despite the seemingly plausible evidence from the present experiment that the time-rate of mutation is affected by temperature under the conditions given, judgment on this matter must be withheld for a while. For, as we shall see later, the work which was being done on the X-chromosome at the same time as, and subsequently to, the work now being described, showed that some unknown factors which ordinarily are not controlled in an experiment may cause significant differences in mutation rates. In the present instance, cultural factors other than temperature itself and conditions caused by it (for example, state of the food) could scarcely have accounted for the effect, since the warmer and cooler cultures were run simultaneously, and were subjected to the same conditions, aside from those dependent on temperature. The possible influence of invisible genetic factors could not categorically be excluded, however, as means whereby the difference in mutation rate might have been caused, since the ancestors of the warmer and the cooler dumpy lines, though they looked alike, might have been differentiated in regard to the proportions they carried of alternative allelomorphs that could not be seen. This seems a rather hypothetical objection, perhaps, specially created to make the difficulty, and the essential agreement between the mutation rates in both "warmer" lots,

vortex and dumpy—although these were known to be different genetically,—does not lend it support. It will therefore be necessary to describe the X-chromosome work in some detail, to show that the point cannot be ignored, and, after this, the latest experiment on the second chromosome will be described, wherein care was taken to avoid this objection.

In concluding the account of the present experiment, we may call attention to the details that were found out concerning the lethals that had appeared. All these lethals were subjected to linkage tests for the determination of their loci, in order to make sure that there was nothing grossly anomolous in the distribution of the mutating loci. The results are graphically presented in figure 1. As we shall later note in the case

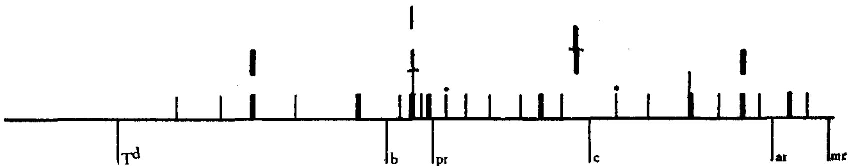

FIGURE 1.—Figure to illustrate mode of distribution of lethals arising in the second chromosome, based on data from the first "balanced lethal" experiment on this chromosome. Genes for visible characters furnishing standards of reference in this mapping are indicated by labelled lines joining from below the horizontal line that represents the chromosome. Genes for lethals are shown by vertical lines above the horizontal line. Lines arranged in vertical order, one above the other, represent genes known to be in identical loci. In the case of each lethal found in the "cooler series" a dot is placed just above the corresponding vertical line. Lethals of the "vortex series" are shown by heavier lines than the others. "Antecedent" lethals found in the course of these tests are shown above, disconnected from the horizontal line. Lethals to the left of T^d could not have been detected. The distribution of the lethals *among* the regions marked off by the "visible" genes shown below is in nearly all cases exact, but their positions *within* their respective "regions" are only approximate.

of the X-chromosome also, the grouping of the lethals here shows characteristics similar to that known for the genes for visible characters in the same chromosome, for, in this case, there is somewhat of a condensation of genes in the central portion of the map, which may be due to a "fore-shortening" of this region. Mutations did occur in all regions, however, and it is evident from the grouping of the lethals that the experiment dealt with the mutation frequency of the collection of genes in the chromosome as a whole, rather than with that of a few selected loci.

As might have been expected, however, mutations did occur in some loci oftener than in others, that is, in a number of cases a given locus mutated more than once (sometimes repeating "antecedent" mutations), and one especially mutable locus gave rise to four separate lethals (in-

cluding one antecedent to the experiment proper). The latter individual gene hence had a mutation rate that borders on the measurable,—about 0.8 percent. Extensive counts were made of the lethal stocks derived from the mutations of the latter gene, but in 6685 chromosome-generations there were no reverse mutations of the mutant back to the normal allelomorph. Had reverse mutation been as frequent as the original type of mutation this lethal could have been used, like variegated corn, for the study of mutation-rate in its own given locus, since the mutations "backward" to non-lethal could have been recognized on mere inspection, by having visible "identifying factors" linked with the lethal. It should be noted that neither in the case of this nor of any of the other loci were we concerned with effects due to asymmetrical crossing over, like the reverse "mutation" of bar eye, since in the present experiment crossing over had been prevented by the "*C* factors."

LESSONS DRAWN FROM MUTATION EXPERIMENTS ON THE X-CHROMOSOME

THE FIRST ATTEMPT TO ESTIMATE THE LETHAL MUTATION RATE

In order to give an understanding of the development of the work on mutation rate it may be explained that the first attempt at an experiment dealing in any way with the conditions of origination of lethals (together with other mutants) consisted of a series of crosses involving sex-linked genes which the writer had his genetics class, at the RICE INSTITUTE, Houston, Texas, carry out cooperatively in the spring of 1918. Each of the 15 students made ten matings, in separate cultures, of pairs of flies of the composition $\underline{w^e v f}$ ♀ and $w^e v f$ ♂. In the formula for the female here, which is heterozygous, the blank spaces below the horizontal line indicate the presence of the normal allelomorphs of the recessive "identifying factors" that are shown in corresponding positions in the formulae of the other chromosomes. The recessive genes w^e, v and f (eosin eye, vermilion eye and forked bristles) lie near the left end, the middle, and the right end, respectively, of the X-chromosome. It will be seen that a lethal occurring in either of the X's of a mother would be evidenced by the absence of all sons carrying, in combination, the two genes lying on either side of the lethal. Among the offspring formed from the cross there would be some females phaenotypically and genetically like the mother and some males like the father (barring mutations), and these could be used for the repetition of the cross in the following generation. In this manner each student was expected to continue each of the ten matings through three generations, by choosing from each culture one pair of flies (the female to be virgin) of composition like the

parents, to continue the "line of descent." Complete classified counts were made of the offspring, so that not only lethals but also conspicuous changes of crossover frequency might be detected.

As no work had yet been done on gene mutation rate it was not known on what scale the work would have to be carried out, but it was hoped that the (theoretically) 300 cultures that were to be raised during the last two generations (after the preliminary test-generation, which was for for the elimination of lethals previously present) might at least give an idea of the order of magnitude of the mutation rate. In addition, it was thought that part of the cultures might as well be utilized for testing out whether or not some one among various agents might be hit upon, which would be able to produce mutations in such high frequency that the effects would be evident on examination of only a few cultures for lethals. With this object in view, each student kept half of his lines as controls and subjected the others, in each of the three generations, to some particular treatment. The same treatment was given to all of the treated cultures of a given person, but each person used a different treatment. The attempt was made to give the maximum treatment practicable, in each case. Among the agents employed were Janus green, methylene blue, lead acetate, alcohol, KNC, low air pressure, high oxygen pressure, heat, cold, and other easily-applied influences that readily suggested themselves as modifiers of vital structures or activities.

The results of the crosses proved negative, in that, after the preliminary "test-generation," no mutations (or very conspicuous changes in crossover frequency) were found at all, with the exception of one lethal that appeared in a control culture. Needless to say, however, not nearly all of the cultures had been properly carried through, and the experiment as a whole, involving the labor of so many inexperienced persons, could not be considered as yielding very critical data. Nevertheless, it did serve as a first try-out of the method, and at least showed that larger and fully reliable numbers would be desirable for establishing even the order of magnitude of the mutation rate; it indicated besides that not even lethal mutations are readily produced *en masse*. Following this experiment, therefore, the writer changed his mode of attack, and began in the fall of 1918, in New York City, synthesizing the elaborate stocks for the balanced-lethal experiment on the second chromosome which has just been described, in the hope of obtaining, this time, much larger numbers that might establish a significant figure for a "control" set of cultures, and possibly for just one "treated" set (the "cooler series") as well.

At about the same time, in the winter and spring of 1918 to 1919 Doctor EDGAR ALTENBURG, at Houston, Texas, was making an attempt to secure significant numbers in a different way, and his attempt was brought to a successful conclusion much sooner.

ALTENBURG'S ESTABLISHMENT OF THE FIRST FIGURE FOR MUTATION RATE—ITS UNEXPECTED MAGNITUDE

In order not to be hampered in the work of obtaining large numbers in a comparatively short time, ALTENBURG used the most direct method possible—that is, that of testing for lethals in the X-chromosome, rather than an autosome (since this requires only one generation instead of two for the test), and, further, determining the presence of these lethals not by a cross involving "identifying factors" but simply by their effect on the sex-ratio. A lethal present in one X of a mother causes the death of half of her sons, and thus results in a 2 ♀ : 1 ♂ ratio in F_1, instead of the usual 1 ♀ : 1 ♂ ratio, no matter what male she is crossed to.

Of course this method suffers from the disadvantage common to all mutation work on the X-chromosome, that each individual which is represented in the "mutation count" has to be tested separately—lethals cannot be accumulated in the X by the balanced lethal method since a male containing a lethal in its X necessarily dies. Furthermore, the counting necessary to determine the sex-ratio takes more time than a mere qualitative determination of the presence of certain classes, which is all that is usually required when identifying factors are involved. And the sex-ratio, when obtained, is subject to a considerable error of sampling, with the resultant occurrence of relatively many "doubtful cases" that have to be followed up in later generations. This following up becomes particularly cumbersome since, in the absence of identifying factors, there is no way of distinguishing by inspection between the offspring that received the questioned lethal and the others, so that a number of the daughters have to be bred in order to be sure of having at least one with the supposed lethal included. On the other hand, owing to the simplicity and directness of the method, it has certain great advantages, in that (1) virgin females are not required in the testing, (2) there is little trouble with poor viability and fertility, (3) results can be obtained from it almost immediately, and there is hence less danger of the experiment collapsing before results are obtained.

The data concerning this experiment have been briefly given in a previous publication (MULLER and ALTENBURG 1919), but they require review here. The flies were divided into about 75 "lines," each of which

was bred through 6 generations, including the preliminary generation in which the female ancestor of the line was tested in order that any lines containing lethals at the start might be eliminated. (Of course no male ancestor could have had a sex-linked lethal.) In each generation usually not more than one female of each line was tested (since it was desired to insure the independent origin of any lethals found), and this test was at the same time the cross that furnished the flies of that line that were to be bred in the next generation. There were 90 females tested in the preliminary generation and 385 in the other five generations combined, each test involving, as above explained, a count of the number of offspring of each sex. Besides this, there were many crosses necessary in the work of following up those cases in which the sex-ratios did not seem decisive, until it could be determined whether or not a lethal were present. Most of the cases which appeared to give a decisive 2:1 ratio were also tested further, and the lethals thus verified. In this way, by reason of the resolution of the critical cases, the final figures acquired considerable precision.

The definitive data were secured before the summer of 1919. They showed that, among the 385 females tested and known to have come from parents that had not received lethals, 13 carried lethals themselves; in none, however, were there any "visible" mutations detected. Since each female carried two X-chromosomes, this meant a rate of lethal mutation of 13 in 770, or 1 lethal in about 60 "X-chromosome-generations"—in other words, nearly 2 percent of mutation.

This figure, the first real figure ever obtained for the rate of gene mutation in a chromosome, was startlingly high—far higher than most Drosophila workers would have anticipated. It showed that (at times at least) the rate of mutation was high enough to permit of its being studied quantitatively by individual tests of the X-chromosome, without accumulation by means of balanced lethals. It seemed logical, therefore, to push further the work upon the X-chromosome, in the hope of securing additional significant results.

JOINT CONFIRMATION OF ALTENBURG'S FIGURE, AND THE SECURING OF A PROBABLY SIGNIFICANT EFFECT OF TEMPERATURE ON THE TIME-RATE OF MUTATION

ALTENBURG and the writer thereupon undertook a joint mutation experiment upon the X-chromosome, in the summer of 1919 (at a time when some of the balanced lethal lines containing vortex had already been established and new ones were being established). Part of the results of this experiment also have previously been abstracted, in the same article

as the sex-ratio tests (and part in Proc. Amer. Soc. Zool. 1920); but they likewise are so related to the present work as to require mention here. The aims of this experiment were, first, to act as a check upon the previous one, in order to confirm or contradict the surprisingly high figure for mutation rate there found, and, second, to procure early evidence, prior to any which might be forthcoming from the balanced lethal experiment, concerning the possible effect of temperature upon the rate of mutation. In the sex ratio work on the X-chromosome all the lines had been propagated at a rather warm room temperature (in Texas), and no attempt had been made to apply different conditions.

In the method used in the 1919 joint experiment, a reversion was made to the class work of the spring of 1918, which has already been described. It will be remembered that here the identifying factors, $w^e v f$, and their normal allelomorphs, were used, each culture involving a cross of the type $\underline{w^e v f}$ ♀ by $w^e v f$ ♂. The presence of these identifiers permitted a lethal to be known by an easy "qualitative test"—the absence of all of a given class of sons, and no count was ordinarily required. This gave much more decisive results in the original "test-cultures" and far fewer temporarily doubtful cases, than did the sex-ratio method. It could in addition be determined at once in which chromosome of the female (the paternally or maternally derived) the lethal lay, and even what its approximate location in this chromosome was. Further, the daughters receiving their mother's lethal could be distinguished from the others by inspection, so that to be sure of perpetuating the lethal in the next generation it was not necessary to carry on the simultaneous breeding of any of their non-lethal sisters. This difficulty did arise in the generation following, however, in case the lethal lay in the $w^e v f$ chromosome, for then the lethal-containing daughters ("F_1") of the original heterozygous female were of the homozygous recessive type, and so there was no way of telling which of *their* daughters ("F_2") had gotten the lethal; in this "F_2" generation, then, the supernumerary breeding of unknowns became necessary. Such a difficulty was never encountered, in any of the generations of testing, when the lethal was in the "wild-type" chromosome.

The chief disadvantage of this method, as compared with that involving sex-ratios, lay in the necessity of securing virgin females in order to be sure that the daughters which resembled their mothers phaenotypically were really like them genotypically, and hence suitable for further breeding. This entailed considerable extra labor, but not as much as was saved by the greater definiteness and speed of the lethal determination in the presence of "identifying characters."

The cultures in this experiment were kept in half-pint milk bottles. The "warmer series" were kept in an incubator at 27.0±1°C. The flies in these cultures started hatching 8 days after the parents had been inserted, and the duration of the generations that was allowed here varied from 12 days to about two weeks, that is, the offspring were placed in fresh culture bottles after this length of time had elapsed since their parents had been placed in the old cultures. The temperature of the "cooler series" was differentiated from the other in sign, but not by a quantitatively constant difference, as the bottles were cooled by keeping them in shallow pans of running sea water (at Woods Hole). Thermometer readings showed that the temperature in these bottles averaged about 19.5°C. The offspring started hatching about 12 days after the parents had been placed in the culture bottle, and the generations were allowed to take from 2½ to 3 weeks' time.

In the warmer series there were 517 females tested, in addition to the ancestral females of the preliminary tests for eliminating antecedent lethal mutations. Among these 517 females tested for new mutations, 13 lethals were found and no visible mutations. This rate of 1 lethal per generation in 40 females, or 1 in 80 X-chromosomes (1.26 percent), is obviously closely similar to the rate of 1 in 60 X-chromosomes found by ALTENBURG at the Texas room-temperature, and calculation shows that the observed difference between these two rates is only equal to 1.1 times its own probable error, a difference having, of course, no significance. In the cooler series, on the other hand, 445 females from non-lethal mothers were tested for new mutations, and among these only 5 lethals were found, and no visible mutations. This gives a rate of one lethal in nearly 180 X-chromosomes, per generation—0.56 percent—or less than half the rate in the warmer series.

It may be added that approximately 100 bottles, those in which the further tests of the lethals were being made, were kept at room temperature, and that, in these, two new lethals arose during the course of this testing. The X-chromosomes available for the detection of such new lethals numbered about 100 here, since one X-chromosome of each parent female (the "maternal" one) already carried a lethal.

The difference between the rates of mutation per X-chromosome, per generation, in the warmer and the cooler series (1.26-0.56 = 0.7 percent), was then compared with its own probable error as calculated by means of the formula previously given $(0.6745\sqrt{P/(n_1 \cdot n_2)})$. Here we have $P = 18$, while n_1, the total count of X-chromosomes in one of the series, is 2×517, or 1034, and n_2, the other count, is 2×445, or 890. This reckon-

ing gave a probable error of 0.3 percent. Thus it turned out that the difference (0.7 percent) was 2.35 times its own probable error. If the rates of mutation per X-chromosome, per generation, under the two conditions actually employed, had really been alike, a difference of this magnitude, in this "expected" direction, would have occurred only once in 18 trials (and a difference of this magnitude in either direction once in 9 trials). This is obviously far from a convincing result (as has always been realized), yet it does give rise to a certain moderate presumption, or "probability," as termed in the original note, in favor of an effect having been produced, and the above numerical value of this probability cannot be reconciled with a criticism which has been made that the "data are clearly not statistically significant."

If now, instead of calculating the rate of mutation per X-chromosome, per generation, we reckon it per X-chromosome per day, month, or any other unit of time, we find a difference larger than the above between the rates in the two series (relative to their own values), since there were more generations in the warmer than in the cooler series, in a given time. On such a basis, we find 2.96 percent of mutation, per X-chromosome, per month, in the warmer series, and 1.06 percent in the cooler series, the difference here being 1.9 percent. The probable error of this difference proves to be 0.63 percent, so that this difference is 3 times its probable error. Such a difference would only occur, in the given direction (expected for most heat effects), in one trial out of 48, if the rates per month were really alike; such a difference, in either direction, would occur in one trial out of 24 if the rates per month were alike.

It is of interest to note that, in contrast to the difference in rate of mutation indicated between the warmer and the cooler series, no evidence of any significance was obtained for a difference in rate between male and female, for, of the 18 lethals which the females of the two series possessed, 7 were in the chromosome they had received from their mother, and 11 in the chromosome from their father. The 100 bottles at room temperature, which gave evidence only on the mutation rate in the "paternal" chromosome (since the other already contained a lethal), yielded, it may be recalled, 2 new "paternal" lethals, so that altogether there were 13 lethals in 1062 paternal chromosomes, or 1 in 82 (1.2 percent) to be compared with 1 in 137 maternal chromosomes (0.73 percent). A difference of this magnitude would occur somewhat oftener than once in 4 trials if the rates were really alike.

Before further experiments on the effect of temperature could be completed (and while the first balanced lethal experiment was being

carried on) the writer undertook to make an extended study of the possible identity and the positions of the loci involved in the mutations already obtained in this joint X-chromosome work. It was easy to map the loci approximately, by noting the relative amounts of numerical deficiency in the various crossover classes. The establishment of identity or non-identity of the loci of any two of the sex-linked lethals found to lie in the same region presented greater difficulty, since two such lethals cannot be crossed together (owing to the inviability of males with a sex-linked lethal). Direct evidence of identity of the locus of two lethal mutations in the X-chromosome can, in fact, be obtained only when both prove to be allelomorphic to the same non-lethal "visible" gene, to which both can be crossed separately.

Such a finding of mutual allelomorphism was obtained in the case of two of the independently arisen lethals. One of these was completely recessive to normal but threw "broad"-looking females after being crossed to "broad" winged males, and the other gave a somewhat "broad" wing and body in heterozygous condition (when with the normal allelomorph), and gave a lethal combination with "broad," the pupae becoming black and dying at an advanced age. These lethals, then, must both have been allelomorphs of "broad" and therefore of each other, but *different* allelomorphs, and the locus may be a relatively mutable one. This is especially probable because a visible mutation in the same locus, giving an extremely "broad" wing (more extreme than "broad" but not as extreme as the previously known allelomorph called "short") occurred in one of the secondary cultures in which the lethals from the main experiment were being tested out. Since these various allelomorphs are different from one another, the high mutability of this locus might be of a different kind from that in variegated corn or in DEMEREC's mutable races of *Drosophila virilis*, where, supposedly, "gene-elements" that have previously mutated are merely being sorted out. There is, moreover, no reason to believe that the apparently high mutability of this locus is connected in any way with the fact that the two mutant genes found here in the main experiment were lethals, since at least three different non-lethal mutations have also been observed to have occurred in the same locus (counting broad, short, and the new mutant above referred to). The finding, in such a locus, of two lethal mutations which, with the three visible mutations, form a series of decreasing viability and of simultaneously increasing somatic effects, indicates rather that the lethal mutations are essentially similar in nature to the others.

Another lethal proved to be an allelomorph of the previously known

visible gene for "facet" eye. Unlike the other lethal allelomorphs of facet, previously known, which have all been designated as "notch," it did not cause the "notch wing" effect. Four of the other lethals, though not allelomorphs of known visible genes, were themselves on certain occasions "visible," that is, a male bearing the gene managed to hatch in a small percentage of the cultures of the given type. One of these four genes, located between scute and prune, caused a tendency to "cloven" thorax in such males; another, between echinus and cut, gave an extremely "diminutive," sterile male; the third, between vermilion and miniature, caused very weak-looking males with characteristically "flimsy" wings and the fourth, between scute and prune, resulted in "collapsed" wings and in leg abnormalities.

The loci of all the lethals studied were (except for the two allelomorphs of broad) probably all different from one another; certainly most of them were, as was proved by tests that showed them to lie between different "visible" genes. The arrangement of the loci of these lethals, as approximately determined in relation to those of standard, visible mutants, is shown in figure 2. It will be noted that about half of them are concentrated

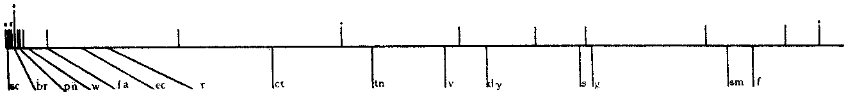

FIGURE 2.—Figure to illustrate mode of distribution of lethals arising in the X-chromosome, based on data from the first temperature experiment on this chromosome. Mode of representation as in figure 1, except that map is drawn to one and one-half times the scale there used.

in the very short region (1.5 units of the $70\pm$) to the "left" of white. This agrees with the similar clustering that has been found in the case of the visible mutant genes in this chromosome, and may merely depend on a "foreshortening" of the map of this region, due to a lower frequency of crossing over here. It was in this region, therefore, that the determination of non-identity of the loci was less accurate in some cases.

It might be objected that all this work of mapping the lethals, determining their allelomorphism, and their possible somatic effects, was a digression from our main object of studying the mutation rate. It was important, however, that such studies be made in one experiment at least. For it was thereby demonstrated (1) that most of the mutations had occurred in different loci; (2) that these loci were grouped in a fashion similar to that known for "visible" mutant genes; (3) that the loci in some cases coincided with those of visible genes; (4) that the allelomorphs in such cases might form a graded series; (5) that there were some semi-lethals which connected lethals and visibles and indicated that there was

no absolute distinction between the latter in regard to the kind of effects produced. All this was desirable in order to show that in such experiments as these we are not merely dealing with a few anomalous genes, that are mutating repeatedly and in some peculiar way, but with chance-given samples of many genes, the mutations of which may probably be taken as representative of mutations in general, including "visible" mutations.

COMPARISON OF DATA OBTAINED ON MUTATION RATE IN THE FIRST AND SECOND CHROMOSOMES

The difference in rate of mutation, apparently associated with temperature, was in the same direction in both the experiments involving temperature differences which have thus far been reported. The results on the second chromosome, though presented first, were, it may be recalled, obtained later than those of the joint experiment on the X-chromosome, and so they could not be taken into account in the preliminary note previously referred to. We may here, however, consider them in combination with the results on the X. For this purpose a special mode of reckoning will be required; it will not suffice simply to make a total of the data in two experiments performed in a different way on different objects. Nor will any unweighted average of the results in the two experiments exhibit the full significance of the combined data.

One valid method of obtaining the most informative figure possible concerning the effect in the combined experiments is as follows: First, express the difference found between the two series in terms of its own probable error as a unit, in the case of each experiment separately. This puts the results of the two experiments into comparable terms. For the X-chromosome, it will be recalled that the difference in mutation rates per chromosome-generation was 2.35 times its probable error, and for the second chromosome 1.6 times its probable error. Now, the chance of obtaining a combination of results at least as improbable as this combination is the same as that of obtaining, in a single experiment, a result equal to the square root of the sum of the squares of these individual values. Making the latter calculation (by taking $\sqrt{(2.35)^2+(1.6)^2}$) we get the figure 2.84. The chance of obtaining, in an individual experiment involving random sampling, a result this many times its probable error is 1 in 17+, or if we consider the direction of the difference as specified, 1 in 35. In other words, there would have been only 1 chance in 35 of getting a combination of differences as "improbable" as that observed, and in the direction of an increase accompanying heat, if the temperature difference, *or some agent accompanying it*, had not really affected the mutation

rate per chromosome, per generation, in *at least one* of these two experiments. Such a result would ordinarily be regarded as furnishing moderately strong evidence for an effect.

The same method of reckoning may be applied to the figures for the mutation rate as measured in terms of "chromosome-months." In the first chromosome the difference was 3 times its probable error, and in the second chromosome almost the same—2.9 times. Calculating as before ($\sqrt{3^2+(2.9)^2}$) we find that the probability of such a combination of differences is the same as that of a single difference 4.15 times its own probable error. An event as improbable as this would occur in only one trial out of 197, regardless of the direction of the difference, or in one trial in 394, in the given direction. It may therefore be considered as "proved" by this work that, in at least one of these two experiments, there was a difference in the time-rate of mutation between the warmer and the cooler series brought about by some cause other than the fluctuations inherent in simple random sampling. It was difficult to believe that the cause could be other than temperature, acting either in a "direct" or in some indirect fashion, since the cultures in the warmer and cooler series had been sensibly alike in other environic respects and it was scarcely to be expected that such chance invisible gene differences as might have been possible here would influence the general mutation rate throughout a chromosome.

There was, however, one conspicuous feature of the work which gave rise to doubt and conjecture on the latter questions. The mutation rate for the X-chromosome was markedly higher than that for the second chromosome, both in the warmer series, which were kept at almost the same temperature in the two experiments, and in the cooler series. The difference should, seemingly, have been in the opposite direction, since the X is only two-thirds as large as the second chromosome (both cytologically and also as measured by the length of the linkage maps). Allowing for this difference in chromosome size, we find that, in the two warmer series alone, the difference between the mutation rates of the two chromosomes, per unit length—reckoned as 13 in (1034×70), and 24 in (4098×105), respectively—is 5.4 times its own probable error, no matter whether "chromosome-unit-generations" or "chromosome-unit-months" are considered. We must therefore conclude either that the mutation rates in the two chromosomes differ, even when they are subjected to identical conditions—this would be most curious—or else that there were differences in other conditions than temperature (either environic or genetic) that caused the difference in the mutation rates of the two experiments. This latter possibility, though it seems the less remote of the two,

would appear strange enough, and yet one or the other of these two conceptions must be correct.

If, now, it was true that such differences in cultural conditions or in genetic composition as distinguished these two experiments could result in the observed difference in mutation rate between them, might it not be true also that some similar environic or hereditary difference happened to exist, unbeknown to the experimenter, between the cultures of the warmer and the cooler series, in one or both of the experiments? In that case, this condition might have been responsible for the significant difference observed between the two series, and we should be wrong in attributing the effect to temperature. How, then, could the possibility of such an influence be avoided? One obvious way was by a repetition of such "temperature experiments," since of course if a result in the same direction was consistently obtained in enough experiments, the influence of factors other than that which had been consistently varied throughout all the experiments alike would finally be excluded. Meanwhile, however, even before the results of the second chromosome experiment had been obtained, other experiments, on the X-chromosome, had in fact been initiated, in the hope of getting further evidence. These experiments, and those that followed them on the X-chromosome, will now be described, as they had an important bearing on the point here at issue, and showed (by a process of elimination) what features the definitive temperature experiment would have to possess.

THE DISCOVERY OF SIGNIFICANT VARIATION IN THE MUTATION RATE, OF UNKNOWN ORIGIN

During the academic year 1919–20, while the lethals found in the joint experiment of 1919 were being mapped, and the cultures involving the second chromosome were being carried along (but not yet tested), crosses were also being made in preparation for an experiment whereby it was hoped that a much larger, more decisive mass of data might be secured, concerning mutation frequency in the X-chromosome at different temperatures. To this end, the writer synthesized an elaborate X-chromosome stock, which was intended to make the determinations and tests of the lethals still easier and more definite, and especially to facilitate the propagation of the lines from generation to generation, by making it unnecessary to secure virgin females. Non-disjunction, too, was allowed for, so that its occurrence would neither interfere with the lethal determination nor make it necessary to secure virgins. In cultures derived from

these lines, moreover, the "supernumerary breeding of unknowns" would in no event be required.

It would be superfluous to detail the genetic circumstances which, theoretically, were to bring about these results, but the composition of the stock synthesized may be recorded here, as follows:

(odd generations) $\dfrac{y\ w^e\ c_v\ c_t\ v\ g\ f}{s_c\ w^e\ \ t_n\ s_m}$ ♀ × $s_c\ t_n\ v\ s_m\ B$ ♂

(even generations) $\dfrac{y\ w^e\ c_v\ c_t\ \ v\ g\ f}{s_c\ \ t_n\ v\ s_m\ B}$ ♀ × $s_c\ w_e\ t_n\ s_m$ ♂

Some thousands of cultures of these types were bred, in joint work of ALTENBURG and the writer, in the summer of 1920, under purposely varied temperature conditions, but the experiment failed because, under the conditions of rearing at Woods Hole, the males containing the genes of the $yw^ec_vc_tvgf$ chromosome, and to a lesser extent those of the other types, had such low viability, even when no definite lethals were present, that the number of "doubtful" cases arising became too large to deal with. The work had to be stopped before nearly all these cases could be resolved—a situation which of course destroyed the significance of the entire experiment.

But from the ruins of this experiment there issued suddenly the hope of a much more effective attack upon the problem. For in one of the last cultures examined by the writer a mutant condition called "C_l" was found, in the paternally derived $s_ct_nvs_mB$ chromosome of a female, which was at the same time (recessive) lethal in its effect, and prevented nearly all crossing over between this chromosome and its not similarly mutated homologue. This "mutation" appeared to me to offer unexampled technical advantages for further mutation work on the X-chromosome. The reasons for this may be explained in detail, as considerable use has been made of the method involving this mutant chromosome, both in the work herein to be reported and in other mutation studies, to be reported elsewhere.

In the first place, the suppression of crossing over caused by the mutant chromosome is highly advantageous. For the occurrence of crossovers seriously hampers the determination of whether or not a lethal is present (even though crossovers are eventually necessary for determining its locus), since only those character-combinations will be uniformly absent from a count which depend on visible genes that lie in their original arrangement on either side of the lethal, that is, it is for the absence of

given non-crossover types that one must look, primarily. We must make an exception here in the case of such specific crossing over as may be necessary in experiments like the one on the second chromosome, previously described, where a certain "antecedent lethal" has to be removed by crossing over before new lethals can be recognized. Even here, however, the new lethal can be recognized only by the absence of flies homozygous for chromosomes that are non-crossovers in the region of this new lethal. The occurrence of most crossovers, then, reduces the number of offspring available for the lethal determination, making the latter less decisive. In addition, the presence of these crossovers makes necessary a much more detailed inspection of the flies, in order that the presence or absence of the crucial non-crossovers may be ascertained.

Secondly, the presence of a lethal in the same X-chromosome of the female as contains the "factor" preventing crossing over, although it leaves only the other X of the female available for the study of new lethals, causes all counts from females containing a new lethal in this other X to exhibit a 1 ♀ :0 ♂ ratio. For the antecedent lethal in the first X-chromosome will kill half the sons, while the new lethal in the homologous X will kill the rest. On the other hand, females in which a lethal has not arisen in this homologous X will throw a 2 ♀ :1 ♂ ratio. We have, therefore, in cultures of such stock, only to distinguish between a 2:1 and a 1:0 sex ratio rather than between a 1:1 and a 2:1 sex ratio, as in the ordinary case, and the former distinction (involving a kind of "all-or-none reaction") is of course much surer, more definite, and more readily determined, than the latter. All that is necessary, then, in testing for a new lethal in a female carrying C_l, is to see whether any males at all are present among the offspring. One does not, ordinarily, even have to distinguish the "identifying characters" of the males. This makes it possible for the determination of the lethal, in most cases, to be made merely by inspection with the naked eye, or with a hand lens, through the glass wall of the culture vessel, without etherization of the flies being required. It is true that an occasional male will appear, even in lethal cultures, made possible by primary non-disjunction or by the sporadic crossing over that takes place, but such lone males can then be further examined, and the cross can have been made in such a way that they will readily reveal their origin by conspicuous "identifying characters," visible through the glass container.

A third advantage of having one of the mother's X-chromosomes contain a known antecedent lethal, and unable to cross over with the other X, is that this results in only one kind of male offspring being produced

(barring the above-mentioned rare crossovers and non-disjunctional exceptions); as the compositions of the parents can be so arranged that this type of male will be suitable for the daughters to mate with, for the continuation of the experiment, it will then be unnecessary to obtain virgin flies, a procedure that otherwise occupies perhaps a third of the working time.

Fourthly, in the C_l-containing chromosome in question, the "visible" mutant genes present, s_c, t_n, v, s_m and B, afforded excellent "identifying factors," so that it was feasible to use, in the homologous chromosome in which new lethals were to be looked for, a contrasting gene-combination possessing relatively high viability. In this way the number of "doubtful cases" might be reduced to a minimum. As a matter of fact, it was decided to use a $w^{co}fB$ (coral forked bar) X-chromosome in this place in some cultures, and in the others one containing the combination $w^e vf$; the high viability of these had been proved in preliminary experiments. A scheme was arranged whereby, in each line, the $w^{co}fB$ and the $w^e vf$ chromosome exchanged places in alternate generations, as this procedure made it possible easily to recognize any flies resulting from non-disjunction. This scheme of breeding was as follows:

odd generations $$\frac{s_c\ t_n\ v\ s_m\ B\ C_l}{w^e\ \ v\ \ f} ♀ \times w^{co} f B ♂$$

even generations $$\frac{s_c\ t_n\ v\ s_m\ B\ C_l}{w^{co}\ \ f\ \ B} ♀ \times w^e\ v\ f ♂$$

It will be seen that such stock perpetuated its alternating composition, and was always ready for lethal tests, without virginity being specially sought for, provided only (1) that the parents were discarded before the offspring hatched, (2) that non-disjunctional or crossover males did not appear, and (3) that a single heterozygously or homozygously bar-eyed female (according to the generation) was taken, with any of her brothers, to start each culture. No "preliminary tests" were necessary since in the paternal chromosome—which alone was studied—there could originally have been no lethal (or the father would not have existed). If in any culture the existence of a new lethal was discovered, by the absence of males, the daughters having the lethal could be distinguished from the others. They would have the composition $\frac{w^e\ v\ f}{w^{co}\ \ f B}$. These females could then be crossed to any males desired, for further testing of the lethal, as they were necessarily virgin (having no living brothers). As they con-

tained "identifying genes" scattered through their X-chromosomes the cross could be made so that their daughters receiving the lethal could also be recognized, and there need not be any "supernumerary breeding of unknowns" in any generation. But since these $\frac{w^e \; v \; f}{w^{co} \; f B}$ females did not themselves contain the "factor" that prevented crossing over, the families derived from them gave immediate data on the locations of the lethals.

A final highly important advantage lay in the fact that these cultures could be reared in 4 by 1 inch vials, rather than in milk bottles, a feature which allowed the preparation and handling of many more cultures. The use of vials, with their smaller total counts, was rendered possible by the fact that, owing to the absence of crossovers, the proportion of flies which gave evidence regarding lethals was greatly raised, as has been explained, and by the further fact that chromosomes could be employed that allowed the males (if non-lethal) to have a high viability.

All these favorable features very much more than compensated for the fact that twice as many cultures were now needed in order to obtain a "mutation count" of size equal to that gotten previously (owing to the limitation that only one chromosome of the female—the "paternal" one—could be studied). It would have been of interest to compare the mutation rates in both maternal and paternal chromosomes, as before, and the limitation of the count to the paternal chromosome was in this respect a drawback. However, there seemed no reason why the fact that the chromosomes tested had all been contained in the male in the generation preceding the test should work seriously against the obtaining of a significant total count, because the earlier experiment had given at least as high a mutation rate in the paternal as in the maternal chromosome.

In accordance, then, with these favorable indications, the writer synthesized the alternating C_l stock above described, multiplied it many fold, and then carried out tests upon it, at the UNIVERSITY OF TEXAS, in the winter and spring of 1921, during twelve fly generations. Throughout this time all the cultures were kept in the incubator at a temperature of 27°C, as it was desired to obtain definitive data for the "higher" temperature first. In the entire experiment at this temperature, a total of 3935 cultures was examined, representing the same number of tested "paternal" chromosomes. The cultures were of two kinds, inasmuch as in 3438 of them the previous generation had been allowed to occupy the customary 10 to 14 days, but in the 497 other cases the female chosen for testing was one in the mother of which the sperm had been "aged" for

a week or more before the fertilization occurred which produced the fly that was tested. The object of the latter special procedure was to obtain evidence as to whether during such aging mutations would occur.

As culture after culture of this experiment was examined, sons of "regular" type continued to be noted in almost all of them until, after the above number of nearly four thousand cultures had been tallied off, it was found that only four lethals, in all, had appeared among them! None of these lethals happened to be from the "aged" sperm.

The above surprisingly low number of mutations was not caused by any lethals having escaped detection, for there were exceptionally few doubtful cases and all of these were eventually resolved. On the contrary, the above number may in one sense be considered as too high, since 2 of the 4 lethals were almost certainly of identical origin; they occurred in sister flies and had, so far as could be ascertained, the same locus. This appearance of two lethals having a common origin was made possible by the fact that the experiment involved a departure from the principle of breeding only one daughter from each parent culture: instead, an average of 8 daughters were bred from a parent culture, and, in compensation, only 1/8 of these filial cultures were then continued further, in the generation succeeding them; this same procedure was followed out in each generation. It is permissible to do this where pedigrees "within the lines" can be kept, and where the tests are made and recorded in each generation (which cannot be done where the lethals are accumulated in balanced stocks). It involves us, however, in certain difficulties in the computation of the mutation rate, as a somewhat larger probable error is then applicable, due to the resulting correlation between the lethals appearing among sisters. Still, the error is not very much larger than in random sampling, because mutations that appear singly even then usually remain in large majority (BRIDGES 1919, and MULLER 1920).

Taking the figure 4 as representing the number of newly arisen lethals, we find a mutation rate of only 1 lethal in a thousand "paternal" X-chromosomes (including, it will be remembered, in an eighth of these cases, chromosomes derived from sperm that had been aged for nearly the length of a zygote-generation). The difference between this rate of 0.1 percent and the rate of 1.2 percent, obtained from the finding of 13 lethals among 1062 paternal X-chromosomes in the earlier joint experiment (including here even the cooler series), is 8.2 times its own probable error, if we calculate the latter by the usual random sampling formula (namely, $0.6745\sqrt{P/(n_1 \cdot n_2)}$). Even if the error should really be taken as twice as large, then, this is an absolutely decisive difference—despite the com-

paratively small number of mutations involved in each case. And it was obtained, be it again noted, in the face of the higher average temperature at which the later experiment was conducted.

Aside from such differences as might have been caused by the use of vials in place of bottles the cultural conditions in the two experiments in question were closely similar. They were almost certainly more alike, in general, than the conditions in ALTENBURG'S sex-ratio experiment in Texas and those in the earlier joint experiment at Woods Hole—the results of which had nevertheless agreed closely. On the other hand, it was also difficult to conceive of the cause of the difference in mutation rates as having been genetic. For, in the work with C_l, the two classes of fathers used in alternate generations had had a very different origin, and one of them—$w^e vf$—was derived from the very stock that had furnished the recessive chromosome in the earlier temperature experiment. As for the dominant chromosome in that experiment, it had, in part of the cultures, been derived from a very different source from that concerned in the other part, without a difference in mutation rate occurring between the two parts. Thus there seemed to be no consistent genetic difference between the cultures in the C_l experiment and those in the earlier joint experiment, to explain the consistent difference in mutation rate. It should further be noted that the C_l-containing chromosome itself had, prior to the mutation by which C_l originated, been used in the later joint temperature experiment, and that in this also, although too many doubtful cases had arisen for an exact mutation count, there had nevertheless clearly been no dearth of real lethals. The C_l-containing chromosome itself could scarcely have exerted any influence on the mutation rate anyway, because only mutations in "paternal" chromosomes, derived from males not containing C_l, were studied, and these mutations must in most cases have occurred in those males.

So small had been the number of mutations in the experiment with C_l that it was obviously impracticable to pursue the original plan of following this with an otherwise identical experiment in which the cultures were kept at a lower temperature. For no significant difference due to temperature could reasonably be expected in such small numbers. Since, moreover, some unknown environic or genetic difference had been able to cause a significant difference between the mutation rates in the experiments already performed, it might again enter, in case of a repetition of the C_l experiment, to produce a conspicuous effect that might incorrectly be attributed to temperature. Thus the attempt to obtain evidence concerning the effect of temperature had again been obstructed. And it appeared

more important, now, to investigate further, or at least to obtain further corroboration of, those large variations in mutation rate, of unknown origin, that had just been met with.

CORROBORATION OF THE UNEXPLAINED DETERMINATE VARIATION

With the above end in view, it was decided to conduct an experiment which should be largely a repetition, on a greater scale, of the earlier joint temperature experiment, but this time without temperature differences, simply in order to determine whether or not figures for the mutation rate substantially like those gotten in that experiment would again be forthcoming. This new experiment was carried out at the UNIVERSITY OF TEXAS, in the fall and winter of 1921–22. It was in part financed by a grant given by the AMERICAN ASSOCIATION FOR THE ADVANCEMENT OF SCIENCE in 1921.

As in the experiment of 1919, the initial crosses ("P_1" generation) consisted of matings of homozygous recessive eosin vermilion forked ($w^e v f$) females, in pairs, to dominant (in the main, wild-type) males. In the three following generations, in which the definitive lethal tests were made, heterozygous females $\frac{(w^e v f}{}$ or sometimes $\frac{w^e v f}{B}$, as will be explained) were crossed, in pairs, to their triply recessive ($w^e v f$) brothers. There were 28 of the original (P_1) cultures. In those numbered 1 to 26 the eosin vermilion forked females were derived from the "regular" stock having this combination of genes, but in those numbered 27 and 28 they were derived from "$w^e v f C_{IA}$" stock (a stock in which BRIDGES had found secondary non-disjunction to be increased and crossing over diminished). In cultures number 1 to 13, inclusive, the dominant males were of normal stock from Florida. In numbers 14 to 26, inclusive, they were of normal stock from Falmouth. In cultures 27 and 28 the dominant males were bar eyed, otherwise normal-appearing, and were homozygous for "C_{IA}," being derived from the same stock (maintained by perpetual selection and crossing) as their $w^e v f$ mates.

The number of P_2 cultures derived from each of the 28 P_1 cultures is shown in table 1. In all, 678 fertile P_2 matings of the heterozygous females were started. As these females occurred in groups of sisters, there was a chance here for lethals of common origin to occur. In the next generation (P_3), however, not more than one pair was taken from each of the above (P_2) cultures, and in P_4 likewise this system was followed. There were 604 fertile P_3 cultures started and 437 fertile P_4 cultures. The reason for the

drop in numbers in this last generation was chiefly because here pairs were only taken from those P_3 cultures in which the mothers (P_3) proved to have been virgin before the mating of the desired type had been made; otherwise P_4 females of the wrong genetic composition might have been obtained. (In the preceding generations, as can easily be worked out, non-virginity would not have interfered with the testing.) The P_1 cultures were raised in bottles, and all the later cultures in vials. They were kept in the incubator at 26.5±0.5°C, and 14±1 days were allowed to a generation.

None of the 28 P_1 females proved to have contained a lethal, as the sex ratios showed. Among the P_2 females tested there were 4 lethals. Of these, two may have had a common origin as they occurred in the same (paternal) chromosome of sisters, and both were situated very near *W* (the normal allelomorph of w^e), in loci that may have been identical. The others in this generation were of separate origin, and those of subsequent generations must have been so, owing to the breeding methods used. Among the P_3 cultures there were 4 lethals, and among the P_4 cultures 2. In all, 6 of the 10 lethals occurred in paternally derived and 4 in maternally derived chromosomes. Seven of them arose in the series derived from the cross of Florida males, 2 in the series from the Falmouth males, and 1 in the "C_{lA}" series. The details concerning them are given in table 2.

If we include in our reckoning only the P_3 and P_4 cultures, since only in these had the lines of descent been kept separate during the preceding generation, we find 6 lethals in 1041 females, that is, in 2082 X-chromosomes, or 1 lethal in 347 X-chromosomes. This gives 0.3 percent of mutation. The inclusion of the P_2 generation in addition would give the figure of 10 lethals in 1719 females, or in 3438 X-chromosomes. This yields the almost identical result of 1 lethal in 344 X-chromosomes, or, again, 0.3 percent of mutation. It will be observed that this rate is markedly below that obtained in the earlier joint experiment, but noticeably above that in the work with C_l.

If, now, we treat the data as a collection of completely independent samples, using the "probable error of a difference" formula based on "simple sampling" ($0.6745\sqrt{P(n_1 \cdot n_2)}$), we find that the difference, 0.65 percent, between the present result of 0.3 percent and the result (0.95 percent) obtained in the earlier joint experiment as a whole (warmer plus cooler series) is 5.7 times its own probable error (or 3.9 times if only P_3 and P_4 are included), and is hence "significant." The difference between the present result and that of 0.1 percent, found in the experiment in-

volving C_l, is 2.8 times itsown probable error, if the latter is obtained by the method just referred to; accordingly this is "probably significant" also. If, on the other hand, the data cannot legitimately be treated as a collection of independent samples, this in itself implies determinate differences in mutation rate between different groups of the cultures within the experiment, and so we reach the same conclusion: that is, that significant variation in mutation rate is occurring, due either to environic or genetic causes.

Examining now the distribution of the lethals we notice at once that four of the ten occurred in the descendants of one of the 28 original P_1 pairs (number 1), although only one-twentieth of the tested females were derived from this pair. Such a concentration of the lethals within one group seems well beyond the limits of a purely random distribution. None of the four lethals could have originated by the same mutation, although two of them, which were in the recessive chromosome very close to w^e, might be conceived to have been a later result of some "premutation" (see footnote, p. 293) that occurred in the common ancestral chromosome. Even if we rule out one of these on account of this possibility the concentration of separate lethals here is still beyond what would be expected in a random distribution. In the group from P_1 pair number 7 we find a situation that appears similar: three lethals in a total of 141 tested females. Here, however, the first two of the lethals, both occurring in the paternal chromosome of P_2 females, in possibly identical loci, may well have been of common origin. If we count them as one, there still would be two lethals in this group, a rather unlikely occurrence for random sampling in an experiment where lethals in general are so rare. The third generation lethal in this group also might be conjectured to have been related to the others by "premutation," as all of them lay in the dominant chromosome, at loci that may have been the same; hence, although "premutation" is of very questionable occurrence in Drosophila, we cannot use the results from group 7 to prove a tendency to an increased general mutation rate, as we can those from group 1. Nevertheless, these two groups (both from the cross of Florida males) certainly corroborate each other, in indicating that some groups mutate significantly more often than others which have and have had apparently the same cultural conditions and "visible" gene composition.

Selecting just these two groups, we find in them, taken together, 7 lethals in 456 X-chromosomes, or 1 in 65 (1.5 percent), which is a rate that agrees closely with that observed in ALTENBURG'S sex-ratio work and in the earlier joint experiment at the higher temperature. On the

other hand, all the other groups, taken together, showed only 3 lethals (all different) in 2982 X-chromosomes, a rate of approximately 1 in 1000, or 0.1 percent, like that found in the work with C_l. And the present experiment, taken as a whole, showed, as has been remarked, a rate significantly different from that found in either of these two previous experiments. Thus, whatever the cause of the variation in mutation rate may have been, the present experiment confirms the conclusion regarding its actuality.

There were certain important lessons to be drawn from these empirical facts, even though their cause was yet problematical, and while it was even doubtful whether genetic or environic factors were responsible, First, it was obviously inadequate to follow the original plan of obtaining data from "controls," all supposedly under one set of conditions, in one year, and data from a group of "treated flies," reared under a purposely different set of conditions, another year. For elusive genetic differences between the two groups, not reflected in any difference in their known formulae, might have cropped up by mutation, or have been spread through differential breeding, in the course of several months, and these might, for all that was yet known, become the cause of any observable changes in the mutation rate. On the other hand, if the previously found unexplained variations in mutation rate had not been due to such genetic causes, then, whatever the environic influences may have been that had unsuspectedly been responsible for the effects, these too might again arise to differentiate two series of cultures run at different times in our projected later experiments. To be sure of avoiding both possible complications, then, the two series would have to be carried along simultaneously, even though this might entail extra labor. They would have to be so *related*, genetically, that *no possible hereditary differences* between them, known or unknown, could account for a difference found in their mutation rates and so *treated* environically that, aside from the agent in question, *no possible external differences* could account for the result.

Second, the C_l-containing stock, despite the advantages it presented for testing and for propagation of the cultures, could probably not be used to advantage for studying such differences in mutation rate as might be caused by different temperatures, as its ordinary mutation rate was, and might again be, too low. Similar conditions might, however, be found at any time (so far as the experimenter could predict) in any other stock. Hence it seemed desirable to use methods that would, with the same labor, allow still more wholesale testing for mutations than any methods previously devised for the X-chromosome.

After these trials of the possibilities of the X-chromosome, it therefore appeared as though the investigation of such (probably) moderate effects as those of temperature might be prosecuted more effectively by a resumption of intensive work with the second chromosome, where the accumulation method could be used. As we have seen, suggestive results had meanwhile been obtained from the experiment first started on this chromosome. The balanced lethal method, though requiring a considerable lapse of time between the initiation and the winding up of an experiment, had allowed the experimenter, with the aid of an assistant (more of the labor being routine in this method) to raise even larger numbers than had the "C_l" method. In addition, the amount of chromatin, and the probable number of genes involved in each chromosome tested, was nearly half again as great, since the second chromosome is $1\frac{1}{2}$ times as large as the X. It is true that, in spite of these advantages, the number of mutations found had not been great enough to give the results already obtained the full significance desired. Nevertheless—and these were the most important considerations—the method was found to be capable of being modified so as to become far more productive than it had been before, for a given amount of skilled labor, and, at the same time, it was found to lend itself readily to the securing of adequate controls, in the sense previously explained.

FURTHER MODIFICATIONS AND TESTS OF THE METHOD OF BALANCED LETHALS

ALLOWING FOR GENETIC AND CULTURAL HETEROGENEITY

It might at first sight appear a difficult matter to elude invisible gene differences and secure, for the different series of an experiment, material that would be sufficiently comparable genetically. Even stock that is originally quite homogeneous is, of course, subject to differentiation through mutations, invisible as well as visible. However, it is possible readily to avoid the effects of genetic diversity simply by following the plan of picking the ancestral individuals, for the lines of the different series, at random from the same original lot. For in that case the possible genetic differences between the individuals, that might influence their mutation rate, would be distributed between the two series according to the laws of random sampling, and could not affect the series differentially to an appreciably greater extent than would be allowed for anyhow, when the ordinary probable error formula was applied to the mutations. This effect depended on the fact that in each series there were a great number of lines,

and that only in a negligible proportion of cases did more than one separable mutation occur in a single line.

The differential effect of possible genetic differences on the two series could be still further reduced, moreover, by establishing the lines before the series were separated, and then forming the two series by splitting each original line into two or more divisions, of which half were placed in one series, and the other half—chosen at random from among the divisions of each original line—in the other series. This was equivalent to "grouping" the lines, a practice already in use to avoid preliminary testing, as previously explained, and to then dividing each group equally among the two series. As carried out in the present experiments, the attempt was made to have at least 100 such groups, and to have at least 4 "sister lines" in each group at the start, in order to make sure that two at least survived to serve as tests of each other. In the case of each group of 4 or more lines, then, 2 (or more) were placed in the "warm" series, and the other 2 (or more) in the "cool" series.

In regard to the problem of overcoming the effects of cultural heterogeneity similar considerations applied. If the two (or more) series of the experiment, which were run for comparison with each other, were carried on at the same time and in practically the same place (where vibration, radiation, barometric pressure, etc., were alike, except in so far as temperature itself might affect these), then all the differences in cultural conditions surrounding the flies in the different containers would be classifiable under the following two heads: (1) the differences in temperature itself and in all conditions (for example, state of the food) that are influenced by temperature under the circumstances in which the cultures are ordinarily maintained, (2) "chance" differences—for example, in regard to amount of food, tightness of stopper, etc.—which are independent of temperature and which a culture in one series is as apt to be affected by in a given direction as a culture in the other series. By the very nature of the conditions under the second head, these will tend to become "evened up" amongst the hundreds (or rather, in this case, amongst the thousands) of individual cultures of the two series, like the genetic differences previously postulated, in such a way that our random-sampling formula, when applied to the mutations finally found, will automatically allow for the practically random effects of these agents. This will leave only the agents mentioned under the first head, to account for "significant" differences in the mutation rate, that is, we can then be sure that any "significant" effect has been caused, either directly or indirectly, by temperature itself. Whether "directly," or "indirectly"—through the other conditions that

are somehow themselves affected by temperature—is of course another question, and one that the experiment by itself cannot pretend to answer.

Be it noted that in thus tracing the cause of an observed significant difference down to temperature or its necessarily associated conditions we have avoided the pitfall that lies open in the interpretation of other experiments, in which the cultures either are not carried on at the same time, or are carried on in places the environic conditions of which have not been carefully guarded. For, in either of the latter contingencies, there might have been differences, such as in light, radiation, quality of food, etc., that consistently distinguished the two series, even though these differences were not *causally*, but only *incidentally*, associated with temperature. It is the possible effect of such agents that we may avoid by means of our precautionary measures.

FACILITATING THE FINAL TESTING OF LETHALS

It was found possible considerably to reduce the labor in the final testing, and so to increase the numbers to be tested, by making use of the chromosome containing "curly," which had just been discovered and analyzed by WARD. For curly wings, which is a definitely dominant "visible" mutant, and was then associated with a recessive lethal, lay in a chromosome containing much more effective "C factors" (inhibitors of crossing over) than $C_{II\ L}$ and $C_{II\ R}$. The latter, it will be remembered, allow an appreciable amount of crossing over in the central portion of the chromosome, which lies between their respective regions of influence. For this reason it was necessary, when they were used, to have a lethal or sterilizing gene in each half of the test-chromosome. As no adequate sterilizing gene was available for the left half, star—a lethal—was used there, and when the final tests were made this had to be removed by a rather rare crossing over in order that the presence of new lethals might be detected. But by substituting the lethal, crossover-inhibiting, "curly" complex, for the $C_{II\ L}C_{II\ R}$ chromosome of the balanced stock, it was possible to make the balancing chromosome, and its homologue, each hold together as a unit, so that only one lethal or sterilizing gene was necessary in the chromosome to be tested. It was chosen to use the sterilizing gene, morula, for this purpose, and to eliminate the lethal, star, from the formula, in order that later, when the final tests were made, it would not be necessary to outcross in such a way as to allow crossing over. Then, if no lethal were present, the entire non-crossover combination, $T^d b p_r c a_r m_r$, would be able to manifest itself in homozygous condition, in one quarter of the "F_{n+2}" flies (barring differential viability), whereas

if a lethal were present there would be no such flies at all. Thus the number of flies per culture which gave testimony to the non-existence of a lethal was vastly raised, and, under fair viability conditions, the number of cultures at first recorded as doubtful could be reduced greatly below the number encountered in the preceding balanced lethal experiment.

The new balanced stock accordingly had as the basis of its composition for this work the formula:

$$\frac{T^d \quad b \qquad p_r \quad c \quad a_r \quad m_r}{C_y \, l_{C_yL} \, C_{C_yL} \quad c_n{}^2 \qquad C_{C_yR}}$$

Here C_y represents the gene for curly, C_{C_yL} and C_{C_yR} are the "C factors" preventing crossing over in the left and right half of the chromosome, respectively, l_{C_yL} is the associated recessive lethal, and $c_n{}^2$ is an associated recessive gene for "cinnabar-2" eye color. Flies of this composition appear normal, except for their curly wings, and are very vigorous and fertile. In some cases cinnabar (c_n), an allelomorph of $c_n{}^2$, was substituted for purple eye (p_r) in the upper chromosome; the flies then had the bright red "cinnabar" eye color.

To make the final tests of balanced lines it may be recalled that a single fly (preferably a male) is taken from each line in the "F_n" generation and outcrossed in some way; F_{n+1} flies containing his test-chromosome are then bred *inter se*, and the F_{n+2} are examined for the presence of the multiply recessive flies. Various possible crosses are feasible in the first generation of this test, but the one which experience has proved to be most suitable is of the given male to a female having the following composition, specially synthesized for the purpose:

$$\frac{S \; T^v \quad b \qquad p_r \quad c \; a_r \; m_r}{C_y \, l_{C_yL} \, C_{C_yL} \, p_r \, c_n{}^2 \quad C_{C_yR}}$$

In the "F_{n+1}" from this cross the flies containing the test-chromosome from one parent and the curly complex from the other parent are readily distinguished from the rest by their having the combination: curly wings, non-star eyes, and purple or cinnabar eye color (according to which of these genes lies in their test-chromosome); the other curly-winged flies are star, and at the same time red eyed. In addition, flies will be formed that receive both chromosomes containing the similar "visible" recessive genes, and these will show all six of the corresponding recessive characters. They differ from the homozygotes to be looked for in F_{n+2} by also having star eyes, and by having long (non-dumpy) wings (although they show the vortices associated with dumpy). The apparition of these recessives

in F_{n+1} is valuable as a verification of the composition of the male from the experimental line, for if he is the result of some previous contamination of the line, these combinations will not appear, and the line must be discarded; thus one kind of later "doubtful case" will be avoided.

In choosing the F_{n+1} flies for mating, it is not absolutely imperative to secure virgins (though the youngest looking females should of course be selected), because, in the absence of crossing over, there is no chance for the test-chromosome to become "contaminated," and flies carrying it and curly can again be recognized and bred in the next and in each following generation in case lack of virginity (which will be apparent in the character of the offspring) should have been the cause of the non-appearance of the homozygotes sought. After virgins of the specified type have thus finally been obtained, in some generation or other, the stock derived from them will not only afford a test for lethals, but it will itself constitute a "self-perpetuating" balanced stock in which the lethal can be held indefinitely, without selection being required; this stock can then be used, at the experimenter's convenience, for any further testing of the lethals—including the test of direct crossing with other lethal stocks to determine possible allelomorphism of the different lethals. Thus the elaborate procedure, involving crossing over, etc., that was previously necessary for obtaining a readily useful stock of the lethal, is avoided. For determining the locations of the lethals, however, simple out-crosses of such stocks to stock containing a chromosome without "*C* factors," followed by inbreeding of the non-curly offspring, are necessary.

It will be seen that by means of the above method, although etherization and selection of the F_{n+1} are still necessary, the procedure is, all in all, very considerably simplified. In fact, if the determination of possible "visible" mutants is not an object, most of the examinations of F_{n+2} bottles for lethals can be made without etherization, by inspection of the flies through the glass of the culture bottle. For the six recessive characters will appear in almost inseparable combination, and thus the homozygotes are very easily distinguishable by the naked eye from their curly winged, otherwise normal-appearing sibs. Recessives resulting from non-virginity, and so carrying T^v in place of one of the T^d genes, are also plainly distinguishable, by their much longer wings, from the typical homozygous combination sought.

SIMPLIFYING THE ESTABLISHMENT OF THE LINES

Some time after this improvement was made in the final testing it was found possible to make an even more radical simplification in the pro-

cedure whereby the lines are started. This simplification was gained by an increase of genetic complexity, which, once established, was self-perpetuating.

The chief object in making the crosses and selections whereby the groups of lines were established was to insure the "unity of the source" from which all the test-chromosomes in a given line or group of lines were derived. Hence, a single male, heterozygous for the test-chromosome, had to be mated, and the offspring which showed, by their "identifying characters," that they had received this chromosome together with the required "balancing" chromosome from the female, had to be selected and bred *inter se*—virgins being eventually necessary. When several hundred lines are in question the amount of labor, thus multiplied, may seriously affect the numbers started. A scheme was accordingly devised whereby the selection of the desired virgin F_1 would be carried out automatically, by reason of the death, genetically produced before hatching, of all the undesired zygotes.

In the working of this scheme, advantage was taken of the peculiarities of the race containing "Translocation I," which had been discovered by BRIDGES to have a portion of one of its second chromosomes removed and attached to one of its third chromosomes. A zygote with a defective second chromosome cannot live unless supplied with the "translocated" section on the third chromosome. Thus, by making the undesired second chromosome of the ancestral female a defective one, there was a chance to kill off those offspring that received it, in a certain contingency—namely, when the translocated piece was not inherited with it. As for the other offspring that received this undesired second chromosome of the female, but received the translocated piece on the third chromosome as well, it was arranged to kill them in another way—by means of other lethals that had been placed in the third chromosomes. For this purpose, in the female in question, the third chromosome containing the translocated piece was given two different lethals (Δ and H), between which crossing over was prevented, and the two third chromosomes of the "source male" that was to be crossed with this female were each given one or the other of these same lethals. For these reasons none of the offspring survived that received the undesired second chromosome of the ancestral female. Now the desired second chromosome of this female contained the curly lethal complex. That made it easy to kill off those offspring that received this desired chromosome but received the undesired second chromosome from the "source male," for if the stock yielding the latter were made up in such a way that its undesired second chromosome contained the curly

lethal complex likewise, the offspring having the combination in question would be homozygous for curly and its associated lethal. Thus there would be left alive only the offspring of the requisite type that had received both the desired second chromosome from the female (the curly-containing chromosome) and also the desired second chromosome from the "source male" (the "test-chromosome").

The males and females serving for this cross were obtainable directly from their two respective stocks, without selection, as the ancestral combinations referred to were of a balanced type, such as would automatically perpetuate only their own composition (barring a very rare crossover). The formula of stock "A," from which the "source males" were derived, was as follows:

(stock "A") $$\frac{T^d\ b \qquad c_n\ c \quad a_r\, m_r}{C_y\ l_{CyL}\, C_{CyL}\, c_n^2\, C_{CyR}} \quad \frac{\Delta}{H}$$

Δ ("delta") and H ("hairless") are the third-chromosome lethals previously referred to; they are dominant for certain visible characters, and their loci are very close together.

The formula of stock "B," from which the females for the cross were derived, is as follows:

(stock "B") $$\frac{P}{C_y\ l_{CyL}\ C_{CyL}\ c_n^2\ C_{CyR}} \quad \frac{\Delta He\ T_r}{C_{\text{III}}\ \ l_{\text{III}\,1}}$$

Here P ("Pale") represents the *absence* of the piece of the second chromosome which, when present attached to the third chromosome, is designated as T_r (for "translocation"). C_{III} is a "C factor"; $l_{\text{III}\,1}$ is a recessive "balancing" lethal; *e* ("ebony") is non-essential but would have been difficult to eliminate in making up the stock.

Reviewing this cross briefly now, in terms of the above symbols, we see first that the P-containing chromosome of stock "B" could survive only when accompanied by T_r. But T_r is accompanied by both the lethals Δ and H, either the one or the other of which it will also encounter from the other stock, "A," and which will therefore kill the fly containing it. The P chromosome, hence, never survives the cross. Considering now the curly-containing chromosome of stock "B," we see that this will, through its lethal, kill the zygote which also receives curly from "A." There still remains the possibility of curly from "B" (together with $C_{\text{III}} l_{\text{III}\,1}$) uniting with the "test-chromosome" from "A" (and with either Δ or H); this is the combination required; it has, so far as its second chromosomes are concerned, precisely the formula previously presented as desirable for flies of experimental balanced lethal lines.

To start the lines, then, it is accordingly necessary only to collect "A" males and virgin "B" females from the respective stock bottles, in large numbers, and then to put a pair in each vial, to be thrown out before the offspring hatch. Each pair is the ancestor of a separate line, or group of lines. Their offspring are automatically of the right composition, because of the genetic machinery that was at work, and those offspring that occur within a given culture all have their test chromosome derived from a single source-chromosome, and can be bred together.

AUTOMATIC PRELIMINARY TESTS OF THE LINES

Since, now, the test-chromosomes in the above offspring (F_1) do not intentionally contain star, or any other actual lethal, at the start, but only a sterilizing gene (m_r), the multiple recessives homozygous for this chromosome should actually appear, in the next generation (F_2) of the line of cultures, if this generation is reared under fairly favorably conditions (that is, in a bottle, in temperately warm surroundings). There they can be recognized through the glass wall of the bottle, with the unaided eye. An "antecedent" lethal unintentionally included will, however, prevent them from appearing. In this way, by merely growing the second generation with some care, in bottles, and inspecting the latter with the naked eye, the lines can be started with preliminary tests that will guarantee the absence of "antecedent lethals." The system based on the groupings of the lines will thus receive a double check, even "parallel mutations" can then be recognized as such, and the useless carrying along of lethal cultures through an experiment will be avoided.

It is true that the sterilizing gene morula cannot be regarded, theoretically, as completely preventing differential survival, because only the female is sterile. The flies containing all the recessive genes, however, hatch very infrequently in the vials, and then are small and weak. The number of lethals lost through the successful competition of a non-lethal homozygous multiple-recessive male of the type in question with a vigorous but lethal-bearing curly fly must therefore be negligible.

The development of the "automatic complementary stocks," "A" and "B," was the result of over a year's work (1921–1922), since many other schemes of mating and balancing, most of them much more elaborate genetically than the "A" and "B" plan described, were constructed and tried out simultaneously. Various others have also been tried since. The scheme given, then, represents the residue, proved most practicable, of all these various trials.

PROPAGATION OF THE CULTURES

The chief remaining portion of the work in which it was desirable to increase the productivity of a given amount of labor consisted in the sheer rearing of so great a number of cultures as was needed during the course of the "n" generations—the preparation of vials, emptying, transferring, etc. True, this work had, by the balanced lethal system, been reduced to a routine that allowed it to be placed in the hands of assistants, but the time consumed in such labor was so great as to leave the maximum number of lines that could be maintained smaller than was desirable, and much smaller than the number which the investigator, by means of the new simplifications in the crossing procedures, could conveniently establish and test.

Attempts were therefore made to improve the efficiency of the technique of propagation, and a number of features of considerable time-saving value were introduced, which it would scarcely be in place here to describe at length. Thus, a much readier method of distribution of the food among the vials, through a funnel, was introduced, and the food was made easier to handle, cheaper, and more suitable for the flies, by the substitution of an equal volume of 50 percent karo (aqueous solution) for half of the banana. The latter modification had already been introduced in the first experiment, and likewise the one of *spraying* the cultures *en masse*, with a yeast suspension, and of sprinkling them with confetti in place of inserting slips of paper. Such petty modifications as the substitution, for labels, of transferable tags attached to rubber bands, that were passed down from culture to culture in each line, also became of importance, when multiplied by the thousands of transfers made.

In spite of these and numerous other innovations, which, taken together, have about tripled the number of cultures possible, the problem of propagation of the lines still remains the most difficult one. It was for a time thought that this had been largely solved by the construction of concrete frames, in each of which 49 vials were embedded, and from which, after anaesthetization *en masse*, by cold, a single (fitted) cover, containing the flies in their respective pits, could be removed; all the flies of the 49 cultures at once might thus be transferred directly to 49 freshly prepared vials of culture medium, similarly embedded in another frame. Further experience has shown, however, that in occasional vials the flies will not yet be numerous enough for transferring, though all the others have long been ready; again, on some occasions, the flies may not fall into the cover pit in sufficient numbers. Thus more experimental work will be necessary

before we have a really feasible method of this general type. It is hoped that others interested in Drosophila cultivation will join in the endeavor to produce something practicable along these lines, as it might be of use also in the cultivation of stocks and in some other types of work.

FURTHER EXPERIMENTS

It is the object of the present paper to review only those experiments of the author, and that recorded work of others, which helped towards the ultimate attainment of an answer to the question whether temperature can somehow affect mutation rate. An account of the mutation work of the next three years, 1922–25, will, therefore, not be presented here. It did, however, play a rôle in the development of the final attack, as the considerable practice in balanced lethal work obtained then confirmed the practicability of using the automatic complementary stocks, "A" and "B," and in addition gave various important hints as to their use, which served to insure the carrying through of the later experiment. One such lesson drawn was that of the necessity of making the tests for lethals at a temperature not more than moderately high (about 25°C), and under not rapidly fluctuating degrees of humidity. Failure to have either one or the other of these conditions observed will lead to a very low viability of the multiple recessives that are sought for—a circumstance that caused the partial collapse of one extended experiment before these requirements were realized.

It may be explained that the reason that the work carried on by the writer, during these years, was in the main not directed at discovering the effect of temperature, was because much better physical facilities for such work were expected in 1925, with the completion of a new biology building containing a refrigeration plant, constant temperature room, and considerable incubator space. In the older building, it would not have been possible to insure the maintenance of nearly all the lines called for at one constant temperature and certainly not at two differing constant temperatures. Accordingly, in view of the amount of labor and expense that each mutation experiment entails, it was felt worth while to wait until the opportunities were optimal. Meanwhile, the experiments that were carried on were mainly directed at the related problem of the effect, on the mutation rate, of "age"—that is, of those internal conditions which change with the rotation of the reproductive cycle. The connection between this problem and our present one has previously been referred to. The results of these experiments, some of which are still in progress, must be published separately.

It should be mentioned that during this period one experiment involving temperature differences was undertaken. This was done, however, in collaboration with Professor F. B. HANSON, the cultures being carried on under his direction, with adequate facilities, at WASHINGTON UNIVERSITY. This experiment involved features which make it more suitable to report separately, or in connection with the experiments involving age differences. The omission of the results here will, it may be stated, in no way militate against the acceptance of the present data, or hamper their critical consideration, because in the joint work the temperature influence was applied in an entirely different way, and the results could not, and do not, either invalidate or confirm those of the present experiment. Meanwhile, the author wishes here to express his appreciation of Professor HANSON'S kindness in agreeing to this grouping of the reports.

THE LATEST TEMPERATURE EXPERIMENT

THE CONDUCT OF THE EXPERIMENT

With the approaching provision of the physical equipment needed, and attainment of facility with the improved breeding methods that had been evolved, it was decided to undertake an experiment which would considerably surpass, in numbers of chromosome-generations finally tested, any that had been previously performed, in order that, if it were at all possible, the question at issue might finally be settled decisively. At the same time it was desired to alter somewhat the method of application of the differential condition (temperature), so that certain more detailed information concerning the incidence of its effect (if any) might be secured.

In accordance with these plans, in May, 1925, 106 (fertile) pairs of flies, derived from the complementary stocks, "A" and "B," were placed in separate culture vials, to form the start of 106 groups of lines, numbered consecutively. In the next two generations, the descendants of each pair, without selection or etherization, were divided at random among 8 cultures, tagged with the given number of their original group, and lettered from A to H, respectively. In each group the appearance of some multiple recessive homozygotes, in some following generation, was noted; this proved that none of the groups of lines originally contained a lethal. The lines marked A to D were then chosen to form the "cooler" series, and those marked E to H, the series treated with a relatively high temperature.

It was intended to keep the cooler series continually at a temperature of 19°C, but as the new building was not ready as soon as expected these

cultures could not uniformly be kept as low as this for the first two generations following the separation of the two series. By dint of special efforts, however, it was managed to keep their temperature within about 2°C of this figure for this period, and after that, when they had been transferred to their new quarters, the temperature of these cultures varied, with rare exceptions, less than 1°C from 19°. Approximately ten days after the flies of this series had been transferred to fresh cultures, they were thrown out and discarded; a sufficient number of their offspring had, in the great majority of cases, hatched 10 or 11 days later (that is, 20–21 days after the parents had been put in), and these were then transferred to a fresh culture again, in which the cycle was repeated.

In April of the following year (1926) one male was taken from each of the surviving cooler lines, and crossed in the manner previously described (pp. 331-332), for the final tests for lethals.

The "warmer series" was subjected to a temperature of 27°C—care being taken not to allow it to go over a degree higher because, as WARD has found, this causes the curly complex to undergo appreciably more crossing over. The facilities were adequate, in both buildings, for maintaining the cultures at this temperature, ±1°. The "warmer series" of cultures were not kept at this temperature throughout their life cycle, however, as it was desired to test somewhat more specifically whether warmth, applied to that part of the life cycle more closely connected with the maturation period, would be effective in changing the rate of mutation. This was done in rather crude fashion in the first six generations of treatment (till the end of September, 1925), and with somewhat more refinement after that.

During the first six generations the cultures of the "warmer series" were kept with the cooler ones, at the same temperature as the latter, from the day on which the parent flies had been removed from them until, 10 days later, the offspring hatching in these cultures were transferred to fresh cultures. The latter were then kept at 27° for 10 days, until the flies (now parents) in it were removed, whereupon it likewise was placed in the cool environment and the cycle was repeated. This was not a critical method of applying warmth at a given period in the life cycle, because the flies, during their 10 days in the warm temperature, were laying eggs continuously, and both their egg cells and their offspring, including larvae of all possible different ages, must have been subjected to the warmth, which was accordingly applied, in the case of different individuals, to very different sections of the life cycle. During this portion of the experiment, then, the warmth may be regarded as having been

applied at practically all stages, but for only half the length of the life cycle. It will be noted, however, that nearly all the offspring were derived from eggs that had been subjected to warmth for at least a short time—generally a long time—before laying.

During the last 10 generations of treatment the warmth was again applied over about half (5/11) of the duration of the fly's life, but it was timed in such a way as to be limited somewhat more nearly to a certain portion of the life cycle, in each generation. This was done as follows: The parents were allowed to stay only four days in the culture from which the offspring were to be taken for continuance of the line. During this egg-laying period the cultures were given the 27° "treatment," and most of the eggs did not get beyond the young larval stage. After these four days the parents were discarded and the vial containing the larvae was placed in the cool room at 19°, with the other series. After about 12 days here those offspring which had by this time hatched into imagos were transferred to a fresh culture vial, which was put in the 27° incubator for 6 days. Then these same imagos (before their offspring had hatched) were retransferred into another culture for the period during which the eggs desired for the continuance of the line were laid; the preceding culture was discarded. Thereupon the cycle was repeated as before, keeping the new cultures containing the imagos (now parents) at 27° for four days, after which the imagos were discarded and the vial placed in the cool room, etc. It will be seen that in this case all the flies that bred had been kept at 27° for a period of 6 to 10 days just preceding the laying of the eggs that were allowed to develop. Aside from this period of warmth, which all the flies underwent, there was only the short period of warmth, 0–4 days long, during which the flies were in the egg and early larval stages. During their later larval development and growth, their pupal metamorphosis, and their earlier adult life, they lived at the same temperature as the cool series.

It will be noted that throughout the above series of breedings the warmer and the cooler series were carried through the same number of generations in a given time, and thus any possible influence of the chronological age of the parents was prevented. Of course the flies hatched sooner in the warmer series, but the time-length of the reproductive cycle that they were allowed to pass through was the same. For the flies of both series were transferred to those cultures from which their offspring were later to be taken, after the same length of time since their parents had been transferred to the cultures from which they themselves had hatched. In fact, these transfers were nearly always made on the same day, in both

series. In the first six generations, when double transfers of the imagos in the warm series were not made, the transfer of that series could not have been made two days later without a possible overlapping of generations, but tests showed that with the intervals and temperatures actually used overlapping did not occur.

It should be stated that in every experiment of this kind cultures occasionally die out, or occasionally they are not ready for transferring at the appointed time. Two cultures were therefore always kept of each line—the fresh culture and the one from which the flies had previously been transferred. If the fresh culture failed, flies were taken from the old culture wherever possible. In the case of the warm series, most of the flies in the older culture belonged to a "younger" generation than in the newer culture, not having been artificially retarded in their breeding by the transferring. Whenever an irregularity of this sort occurred, record was kept of it, so that the maximum total number of generations of the warm series, and the minimum of the cool series, could be computed, the figures presented in the next section, in order to be "on the side of caution" in interpreting the results, represent these extreme values. Such occurrences did not take place often, however, and could not have appreciably affected the mode of influence of the treatment upon the germ plasm of the organisms.

In April and May, 1926, one generation after the tests of the "cooler lines" had been started, the similar tests of the surviving "warmer lines" were begun.

THE RESULTS AND THEIR EVALUATION—THE EFFECTIVENESS OF TEMPERATURE WHEN THE DURATION OF THE GENERATION IS HELD CONSTANT

There were 381 lines of the original 424 belonging to the cooler series that had survived to be tested, representing 6286 "chromosome-generations" (*ln*), or 4191 "chromosome-months." Among these, only 12 lines, all of different groups, were found to contain a lethal; all others yielded the multiple recessives. This is a rate of 1 lethal in 524 chromosome-generations, or 0.19 percent of mutation per generation, for this chromosome. The time-rate is 1 in 349 chromosome-months, or 0.29 percent.

In the warmer series there were 359 surviving lines from the original 424, computed to represent 6462 "chromosome-generations," or 4308 chromosome-months. The number of these lines found to contain a lethal was 31. From these figures we may compute a mutation rate, per chromosome-

generation, of 1 in 208, or 0.48 percent. The time-rate is 1 in 139 chromosome-months, or 0.72 percent.

Of the lethals in the warm series only two sets, of two each, were numbered alike—that is, from the same group,—and only in three of the groups in which a lethal occurred in the warm series did one also occur in the cool series. This grouping of lethals was not greater than would be expected in a chance distribution; in fact, it was somewhat less than the most probable value for the expected grouping, though it did not deviate from the latter more widely than the error of random sampling would allow for. Every group containing one or more lethal lines also contained several surviving non-lethal lines. All lethals were verified by breeding the corresponding stocks derived from the test crosses through at least five generations, under favorable conditions, and examining the cultures in each generation for the multiple recessives. We may take the figure 31 of the warmer series as not too high, therefore, and as not representing the special mutability of a few groups of lines.

The difference between the mutation rates, per chromosome-generation, of the two series, is .29 percent. This is 4.1+ times its own probable error (calculated by the random sampling formula previously given). Such a difference would have only 1 chance of occurrence in 195 experiments, if the rates were really the same, or 1 in 390 if we consider the direction of the change as specified, and the effect may therefore be regarded as "proved," in the sense of having that high probability which passes as proof in most scientific work. The difference between the time rates of mutation is equally significant, since the time : generation relationship was the same in the two series.

Both the latest experiment, and the two previous temperature experiments combined, thus agree in giving convincing evidence that the *time-rate* of mutation varies with the temperature, the earlier results showing that this occurs when the duration of the generations varies naturally (more generations per unit time when warmer), and the recent results showing the same effect *even though* no more generations are allowed at the warmer temperature than at the cooler.

Regarding the results now from the point of view of the mutation rate per generation, it can be concluded from this last experiment that this rate rises when the temperature is increased, provided the absolute time-length of the generation be kept constant. Beyond this, from the earlier experiments, combined, there has been established a fair probability, though not as decisive as the above, that a rise in temperature increases the mutation rate, per generation, *even* when the generations

are allowed to have lengths that vary with the temperature as the rate of development naturally varies (the fly thus breeding at about the same "physiological age"). If this should hold true there would ordinarily be both more generations in a given time and, in addition, more mutations per generation, at a higher temperature, and the effect of temperature on the time-rate would therefore usually involve the product of both these factors.

It will be noticed that not only are the apparent effects on mutation rate of the same sign in all three experiments, as well as of convincing magnitude in certain cases considered separately, but that the intensity of the effect produced is also closely similar, approximating the relation, $Q_{10}=2$ to 3, well known for chemical reactions. Though not so much significance can be attached to the exact magnitude of the increment, nevertheless this agreement is "suggestive."

This apparently high effectiveness of heat was produced in the recent experiment even though the treated flies were, for the most part, subjected to the heat over only a special fraction of their life cycle—including principally the period of maturation or maturity, and to a lesser extent the period corresponding to the rather young larva. Only about half of the flies in about a third of the generations (the first six) were treated in other parts of their life cycle; the pronounced effects of heat observed could, therefore, scarcely all be referable to the relatively few treatments during these other periods. The indications are consequently very strong that heat at one or both of these two *particular periods*, just specified, is effective in increasing the mutation rate. Whether, when applied to other particular portions of the life cycle, the heat would be equally effective, or effective at all, we cannot say from such results, except by way of noting that the effects observed were as marked in the later experiment, when only this period was treated, as in the former, when the whole life cycle was treated. This problem, or a closely related one, is involved also in experiments that deal more specifically with the effect of aging.

INTERPRETATION OF THE FINDINGS, AND GENERAL CONSIDERATIONS

Although these results constitute, in the opinion of the writer, the first demonstration of the effectiveness of any specified agent whatever in influencing the mutations of numerous genes, and probably of genes in general, yet we must be exceptionally cautious in going far from these facts and attempting to draw still more general or remote conclusions from them. The sheer fact of the temperature effect on mutation

is worth having, but by itself it stands as an isolated beam in the largely unseen structure of mutation and gene theory. More results gained by similar methods are badly needed. But, in this connection, perhaps the most hopeful feature of the present data is that they show that mutation is indeed capable of being influenced "artificially"—that it does not stand as an unreachable god playing its pranks upon us from some impregnable citadel in the germ plasm; instead, it can be "moved," and its movements detected, studied and "mapped."

It should be repeated here that we do not as yet have any valid evidence on the question of how direct the effect of temperature upon mutation is. We know, however, that in the case of ordinary chemical reactions, the direct effect of a rise in temperature is in the direction of an increase in the speed of the processes, and that the magnitude of this increase is between about 100 and 200 percent for each 10 degrees centigrade (for ordinary temperatures). We have seen, in the present work, that in the case of mutation rate also the effect of a rise of temperature is in the "positive" direction, and that the magnitude of the effect observed here too seems rather simular to that just stated. These facts, then, certainly suggest that mutation depends primarily on a chemical reaction, and is thus directly affected by temperature; pushing the conclusion further, it would become probable that mutation consists ultimately in changes of structure of the general type conventionally designated as "chemical" rather than of one of the types called "physical," (not to speak of such imaginary types as vitalists might postulate). But these points can certainly not be regarded as critically proved, for changes in chemical reations, dependent on temperature, may in turn cause marked effects on physical processes, and *vice versa*.

We know, for example, that the frequency of the semi-mechanical process of crossing over is, in certain chromosome regions, about doubled by a rise of 10°C, at a certain temperature level, and this may quite possibly be brought about through a primary chemical effect of temperature, that in turn influences some "physical" property like chromosome plasticity. In some similar way it might be supposed that mutation, though itself a "physical" process (that is, not involving changes in intra-molecular attachments), could be influenced by a chemical change or complicated series of changes occurring outside of the genes. If a series of changes was involved, the initial process (the effect of temperature upon which was ultimately responsible for the changes in mutation frequency) might even be outside of the organism itself, since as has before been pointed out, we cannot absolutely exclude such,

possibilities as that a change in composition of the food, or in some other cultural condition, itself somehow dependent on the temperature, secondarily affected the mutation rate. We may return to the point, however, that in the case of any sorts of indirect action such as those pictured above, the facts that the effect of a rise in temperature was positive in direction, and was of apparently the same magnitude as are the direct effects on chemical reactions, would have to be regarded as in the nature of a "coincidence." And coincidences do not form good postulates.

One of the points to be remembered in considering the possible mode of action of any agent in changing the structure of a gene is that we are not necessarily dealing here simply with an alteration in the composition of pre-existing gene material, but we may instead, or in addition, be dealing with some kind of interference, by the agent in question, with the process by which the pre-existing gene forms new gene material. The pre-existing gene may remain unmutated, and the "mutation" may consist in the fact that, for some reason, the new gene material built up at that particular time was not just like the old. In that case, the more rapidly gene growth occurred (that is, the more gene material was formed in unit time) during the time that the "interfering agent" was able to act, the greater would be the number of mutations that occurred. Since an ordinary rise in temperature, during stages when cell growth is occurring, usually increases the rapidity of that growth, this by itself would then lead to a direct effect of temperature on mutation frequency, even if other effective factors remained constant. Evidence on this question might be gained if we found that the effect of temperature on mutation rate varied in direct proportion to the rapidity of gene growth that was going on in the germ plasm at the time when temperature was applied. For example, on this hypothesis, warmth applied to mature spermatozoa should produce no such effect. It was because of these considerations that the series of cultures involving the aging of spermatozoa was carried on, but, it will be recalled, the mutation rate was too low in that experiment to permit the securing of results. Similar work, with the aid of the newer methods, should be more informative.

There is at least one path of indirect action of temperature that might, on *a priori* grounds, have been postulated as a mechanism whereby mutation rate could be influenced, which can be categorically eliminated as a cause of the effects observed in the present experiments; that is, the possibility that the mutation rate was affected through the known effect of temperature upon crossing over. Such an idea may have already

suggested itself to the reader in view of the peculiar relationships found to exist between crossing over and bar eye "mutation," by STURTEVANT, on the one hand, and between crossing over and reddish mutation in *D. virilis*, by DEMEREC, on the other hand. Fortunately for a decision on this point, crossing over could not occur in the present experiments, in those chromosomes in which mutation was looked for. Furthermore, most of the mutations occurred in chromosome regions the crossover frequency of which is affected little or not at all by ordinary temperature changes. The mutations observed, then, were not phenomena of exchange between homologous chromosomes. They may well have been affected, however, by some of the same forces (for example, those exerted in synaptic attraction) as also influence the process of crossing over.

An attack on such questions as the above, also, does not now seem so remote. Whether or not, or how, certain synaptic occurrences are associated with mutational changes in general is one of the topics that may be investigated by modifications of our present methods. An intensive mutation study, in which given conditions, known to affect another process in question (for example, synapsis), are concentrated at crucial stages of the life cycle, could scarcely fail to yield evidence regarding such a point.

In the light of the new "gene-element" conception further and perhaps even deeper problems are raised by the present study. Accepting, that is, for purposes of discussion, this new theory, it is not clear whether the effect of temperature here detected would be due to a greater rate of sorting out of "gene-elements" already heterogeneous in the gene before the experiment started, or to an actually greater rate of "gene-element mutation," or both. Various indications, however, would point to the rate of mutation in whatever are the primary gene particles as having itself been increased. One of these indications is the usual lack of grouping in sister lines of those identically located lethals which were found in most of the experiments,—although the latest experiment on the X here seems to form an exception. Another indication is the finding of apparently as great an effect of temperature on mutation frequency, per generation, in the experiments involving many generations as in those involving few, whereas if merely the rate of sorting out of elements had been hastened the supply of differing elements would have dwindled away in the course of time. On this point too, however, the results are only "suggestive." The methods here used are, however, capable of application to this problem too.

Evolution theory and practical breeding must in part follow in the

wake of mutation study. Evolutionists would doubtless eagerly make use of the notion that mutation happens more frequently, per unit time, and also probably per natural generation, at warmer temperatures. And if this were constantly true it could scarcely fail to be an important factor in the rate of evolution, since mutations seem to be so rare that their rate may often be the *limiting* factor in the rate of evolution, and the latter process will then be directly proportional to the former (other things being equal). But it must be pointed out that the significance of the results here presented, for evolution, must largely depend upon the answer to some of the problems previously raised. Take, for example, the problem last discussed, as to which hypothetical part of the process of mutation has been accelerated: The change in the ultimate gene elements, or in their postulated rate of sorting out (and, possibly, in their differential rate of multiplication) to form manifestly different genes. If the former process has been speeded up, the effect would indeed be important in the long run, and therefore of consequence in evolution; if the lattter process only is involved, the effect might be evanescent, since the rate of supply of new "gene-elements" would not be increased. It must be remembered, too, that "other things" are not equal, in nature, and that other factors (including those of selection) differentiating warmer from cooler climates may, on occasion, be vastly more influential than an effect of temperature upon mutation rate itself. This too, however, is within the pale of investigation.

In practical breeding, any factor should be of importance that can affect mutation rate, even if for only a few generations. The implications of the present study for the improvement of organisms whose germ cells can be subjected to controlled temperatures are therefore obvious, and need not be dwelt upon here further. Conversely, cold might be used as an aid in maintaining genic stability in already standardized races.

In conclusion, it may be repeated that, while the effect of temperature on mutation here observed seems of interest, more special emphasis should perhaps be placed on the opening up of the new methods here set forth, and on the proof that these methods can be used successfully in attacking problems which hitherto have been inaccessible. Temperature is merely one of a great number of conditions—external and internal—the effect of which upon mutation can be studied in various ways. And, as the present work demonstrates, some of these other conditions, or at least one, certainly do exert an influence upon mutation rate far greater even than that of temperature. As to what these conditions, or this condition, consist of, the present experiments give little hint, though

they varied markedly from experiment to experiment. This in itself presents an alluring problem, which likewise seems capable of approach through the present methods. Thus, through attacks of this kind, we may perhaps hope for the study of mutation eventually to pass from its earlier observational and speculative stage to one of quantitative and controlled study, from which exact knowledge, and principles not now to be guessed, may finally emerge. The "factor theory" itself awaited intensive quantitative study before its structure could be soundly established, so did the chromosome theory, and so, probably, will the future theory of mutation.

It may appear as though experiments of this type are too cumbersome to be prosecuted. They are not nearly so cumbersome as they were when the first results were obtained with them, and still better methods are, it is hoped, being developed. Since, however, the methods can be successfully used at all, then, for the very reason that they do require effort, it becomes all the more needful for a larger corps of investigators to step into the work thus provided, to make still further improvements, and to gain further data on the important problems that abound in this new field. Each plant-generation in the earlier work on Mendelian inheritance required a year, and much labor, and an experiment required several years; yet through such work biology made relatively rapid strides. And the mutation work is now only in its early years.

SUMMARY

1. The development is traced of methods of obtaining valid data on the frequency of gene mutations under varying conditions. The methods fall under two general groups:

(a) Tests of the X-chromosomes. These again fall into two subgroups—those involving sex ratio counts of each test-culture, and those involving the determination of the presence or absence, in each test-culture, of certain classes of males from mothers heterozygous for sex-linked "identifying genes."

(b) Tests of autosomes. Here stocks containing balanced lethal or sterility genes may be used, to allow the accumulation of mutant genes with the exclusion of natural selection, and the tests, involving "identifying characters" observed in the second generation, are then applied only to the final test-culture of each of the numerous lines of descent.

2. Explanations are given of the purposes, and of the modes of operation of various special genetic devices that facilitate the establishment of the test-cultures and the making of the tests, so as to render possible the

obtaining of a significantly large body of data. Among these devices may be mentioned particularly:

(a) In the case of work on the X-chromosome, the use of the "C_l" complex, discovered in the course of the work, which makes it possible to detect sex-linked lethals by inspection of the culture vessels with the naked eye, to use vials instead of bottles as culture vessels, and to continue the tests in each generation by simply crossing non-virgin females with any of their brothers.

(b) In tests on the second chromosome, the use of the "curly" complex in the "balancing chromosome," in conjunction with a sterility gene instead of a lethal in the test-chromosome; this likewise makes possible the detection of lethals by naked eye inspection of the culture vessels.

(c) In establishing the initial cultures in work with the second chromosome, the use of "translocation I," together with lethals in the third chromosomes, in special arrangement, to kill off automatically all flies except those of the required composition and thus to insure the "unity of the source" of tested chromatin in each line, or group of lines, of descent.

(d) The method of grouping the balanced lines in such a way that lethals present prior to the beginning of the experiment would be recognized as such when the final tests were made, and the splitting of each group of lines between the two contrasted series of an experiment so as to avoid the effects of invisible genetic heterogeneity.

3. The tests carried out by the aid of these methods demonstrated their adequacy, under "suitable conditions" (see 5), for the attainment of significant figures, in which the error caused by "random sampling" was sufficiently smaller than differences due to "determinate causes." This will be realized on inspection of table 3, pp. 354–5, where the data from all the experiments herein reported are summarized,—comprising a total of over twenty-four thousand definitive test-cultures and a still greater number of chromosomes tested for mutation.

4. The lethals found in some of the experiments were mapped (see figures 1 and 2), and tests to determine allelomorphism were given. While a few more mutable loci were encountered, on the whole the genes were distributed in a manner similar to that found in ordinary work on "visible genes." Gradations between lethals and "visible genes" were found, as well as cases of allelomorphism between lethals and visibles. Thus, detailed study of the lethals has shown that they do not constitute a class genetically different from other mutants, or confined to a few

special loci, but that the mutations giving rise to them may legitimately be used as an index of gene mutations in general.

5. The results obtained in different experiments show beyond question that, instead of mutation proceeding at a fixed rate, as might have been supposed, it is exceedingly changeable in its frequency, and, in fact, variations of the order of 1000 percent can follow from unknown causes that invisibly differentiate experiments apparently similar, and involving only ordinary cultural conditions. In the light of these findings, it is probable that the failure of attempts of previous investigators to prove any effect of drastic external conditions upon the occurrence of mutation has been due rather to lack of refinement of their methods (which utilized only the exceedingly rare definitely "visible" mutations as an index of mutation rate) than to actual non-effectiveness of any of the agents employed.

6. It is found that, at the higher levels of mutation frequency encountered in the course of these experiments, it is practicable to use the X-chromosome in studies of mutation rate, but, at the lower levels, such as were more often encountered, the most highly improved methods involving balanced stocks are usually desirable.

7. Results from the earlier temperature experiments on both the X and the second chromosome agree in rendering it fairly probable, though by no means certain (since genetic heterogeneity was not so well allowed for) that the rate of mutation, *per generation*, rises with increase in temperature, even though the generations at the higher temperature are allowed to occupy a correspondingly shorter time (the generations at both temperatures being allowed to succeed one another at rates about proportional to their maximum rates for those temperatures).

8. The experiments just mentioned indicate, with a much higher probability, that the frequency of mutations, in a given time,—that is, the *time-rate* of mutation—rises with increase in temperature, when the generations thus succeed each other at the rates "natural" for the respective temperatures.

9. The latest experiment on the second chromosome, employing improved methods that made possible the use of a much larger number of cultures and also the definitive elimination of both genetic and cultural heterogeneity as possible agents in a differential effect, yielded decisive evidence of a rise in mutation rate, dependent upon increase of temperature, when the duration of the generations was caused to be the same at the two temperatures. Under these circumstances, then, both the time-

rate of mutation, and the rate per generation, is known to be affected by temperature.

10. During the greater part of the time during which the experiment just referred to was carried on, the flies of the "warmer series" were kept at the higher temperature only for the later portion of their imaginal life (for 6 to 10 days) and in relatively early larval stages (for 0 to 4 days); for the rest of their lives (for 11 days) they remained at the same temperature as that at which the "cooler series" was kept constantly. It is therefore likely that warmth is effective in influencing mutation when applied specifically to the stages mentioned.

11. It should be noted that both the direction of the effect of temperature on the time-rate of mutation, and its approximate magnitude, are the same as in the case of its effect on the time-rate of ordinary chemical reactions.

12. Possible interpretations of the findings concerning temperature are discussed, and their bearings on other topics are pointed out.

13. It is believed that the methods by which these results have been obtained open up a new field of genetics—the quantitative study of gene mutation, as occurring throughout one or more entire chromosomes under purposely varied conditions.

TABLE 1

Distribution of cultures in the 1921–1922 mutation experiment on the X-chromosome.

NUMERICAL DESIGNATION OF P_1 PAIR	STOCK OF P_1 MALE	NUMBER OF P_2 PAIRS FROM CORRESPONDING P_1 PAIR	NUMBER OF LETHALS IN P_2	NUMBER OF P_3 PAIRS FROM CORRESPONDING P_2 PAIRS	NUMBER OF LETHALS IN P_3	NUMBER OF P_4 PAIRS FROM CORRESPONDING P_3 PAIRS	NUMBER OF LETHALS IN P_4
1	Fla.	36		32	3	19	1
2	"	19		16		9	
3	"	45		36		22	
4	"	5		5		2	
5	"	33		24		23	
6	"	6		5		2	
7	"	60	2	50		31	1
8	"	7		5		3	
9	"	27		23		15	
10	"	50		48		39	
11	"	27		26		22	
12	"	6		4		4	
13	"	19		16		10	
Total Florida		340	2	290	3	201	2
14	Falm.	8	1	4		2	
15	"	10		10		8	
16	"	26		25		22	
17	"	30		27		17	
18	"	30		29		26	
19	"	30		28		17	
20	"	17		17		11	
21	"	9		8		5	
22	"	4		4		3	
23	"	10		9		6	
24	"	46		45		43	
25	"	23	1	22		19	
26	"	24		24		23	
Total Falmouth		267	2	252	0	202	0
27	C_{IA}	49		43	1	23	
28	"	22		19		11	
Grand Total		678	4	604	4	437	2

TABLE 2

Lethals found in the 1921-1922 mutation experiment on the X-chromesome.

DERIVATION OF LETHAL-CONTAINING CULTURE[1]	GENERATION OF FEMALE FOUND HETEROZYGOUS FOR THE LETHAL	APPROXIMATE LOCATION OF THE LETHAL	CLASSIFICATION OF CHROMOSOME IN WHICH LETHAL AROSE
1.7	P_4	near w^e	Paternal; recessive
1.8	P_3	slightly right of w^e	Paternal; recessive
1.27	P_3	near W	Maternal; dominant
1.28	P_3	slightly left of V	Maternal; dominant
7.4	P_2	very near W	Paternal; dominant
7.27	P_2	very near W	Paternal; dominant
7.34	P_4	slightly right of W	Maternal; dominant
14.1	P_2	near v	Maternal; recessive
25.13	P_2	slightly left of F	Paternal; dominant
27.26	P_3	near w^e	Paternal; recessive

[1] First number given is that of ancestral P_1 pair; second number denotes which P_2 pair from that P_1 pair the lethal culture consisted of, or was derived from.

TABLE 3

Chronological summary of mutation experiments reported in the present paper.

	EXPERIMENTERS	TIME OF EXPERIMENT (EXCLUSIVE OF PREPARATION OF STOCKS, MAPPING OF LETHALS, ETC.)	PLACE OF EXPERIMENT	CHROMOSOME TESTED	TYPE OF CULTURES	APPROXIMATE TEMPERATURES	TOTAL NUMBER OF "TEST CULTURES" (EXCLUSIVE OF PRELIMINARIES ETC.)
1	Muller with class	1918 (spring)	Houston, Texas	X	heterozygous w^e v f	warm, room (25° ±)	results indefinite (100 to 200)
2	Altenburg	1918–1919 (winter and spring)	Houston, Texas	X	wild type (sex ratio tests)	warm, room (25° ±)	385
3	Altenburg and Muller	1919 (summer)	Woods Hole, Mass.	X	heterozygous w^e v f	warm(27.5°)	517
						moderate, room	(100)
						cooler (19.5° ±)	445
4	Muller	1919 (spring) to 1920 (fall)	New York City and Austin, Texas	second	balanced lethal	warm (26.5°)	4098
						cooler (18° ±)	726
5	Altenburg and Muller	1920 (summer)	Woods Hole, Mass.	X	complex heterozygous	warm (27.5°) and cooler (21° ±)	results indefinite (2000+)
6	Muller	1921 (winter and spring)	Austin, Texas	X	involving "C_l" inversion	warm (27°)	3935
7	Muller	1921–1922 (fall and winter)	"	X	heterozygous w^e v f	warm (26.5°)	1719
8	Muller	1925 (spring) to 1926 (spring)	"	second	modified balanced lethal	warm 27°)	6462
						cool (19°)	6286
9	Total of definite counts						24,573

TABLE 3 (Continued)

	NUMBER OF CHROMOSOMES TESTED PER CULTURE	NUMBER OF CHROMOSOME GENERATIONS TESTED	NUMBER OF LETHALS FOUND	NUMBER OF CHROMOSOME-GENERATIONS INCLUDING ONE LETHAL	NUMBER OF CHROMOSOME-MONTHS INCLUDING ONE LETHAL	DIFFERENCE BETWEEN TIME-RATES OF MUTATION IN WARMER AND COOLER SERIES ÷ ITS PROBABLE ERROR	RESULTS REPORTED ON PAGES
1	2		(1)				307
2	2	770	13	59	30		309
3	2	1034	13	80	34	3.0	311-314
	(1)	(100)	(2)				
	2	890	5	178	94		
4	1	4098	24	170	85	2.9	301-302
	1	726	2	363	323		305-306
5	2		"numerous" but indefinite				318
6	1	3935	4	984	430		321-322
7	2	3438	10	344	155		324-326
8	1	6462	31	208	139	4.1	341-342
	1	6286	12	524	349		
9		27,639	114	(242)	(138)		

LITERATURE CITED

ALTENBURG, E., 1919 A quantitative study of mutation in the X-chromosome of Drosophila. (unpublished paper).

BAUR, E., 1926a Untersuchungen über Faktormutationen.
I. *Antirrhinum majus* mut. *phantastica*. Zeitschr. indukt. Abstamm. u. Vererb. **41**: 47–53.
1926b Untersuchungen über Faktormutationen.
II. Die Häufigkeit von Faktormutation in verschiedenen Sippen von *Antirrhinum majus*.
III. Über das gehäufte Vorkommen einer Faktormutation in einer bestimmten Sippe von *Antirrhinum majus*. Zeitschr. indukt. Abstamm. u. Vererb. **41**: 251–258.

BRIDGES, C. B., 1919 The developmental stages at which mutations occur in the germ tract. Proc. Soc. Exp. Biol. Med. **17**: 1–2.
1923 The translocation of a section of chromosome II upon chromosome III in Drosophila. (Abstr.) Anat. Record **24**: 426.

DEMEREC, M., 1926a Reddish—a frequently "mutating" character in *Drosophila virilis*. Proc. Nat. Acad. Sci. **12**: 11–16.
1926b Miniature-alpha—a second frequently "mutating" character in *Drosophila virilis*. Proc. Nat. Acad. Sci. **12**: 687–690.
1927 Magenta-alpha—a third frequently "mutating" character in *Drosophila virilis*. Proc. Nat. Acad. Sci. **13**: 249–253.

DUNCAN, F. N., 1915 An attempt to produce mutations through hybridization. Amer. Nat. **49**: 575–582.

EYSTER, W. H., 1924a The nature of genes to form multiple allelomorphs. (Abstr.) Anat. Rec. **29**: 134.
1924b The effect of environment on variegation pattern. (Abstr.) Anat. Rec. **29**: 134–5.

GUYENOT, E., 1914 Action des rayons ultra-violets sur *Drosophila ampelophila*, Low., Bull. Scient. France Belg. **48**: 160–169.

HAYES, H. K., and BREWBAKER, H. E., 1924 Frequency of mutations for chlorophyll-deficient seedlings in maize. Jour. Hered. **15**: 497–502.

MANN, M. C., 1923 A demonstration of the stability of the genes of an inbred stock of *Drosophila melanogaster* under experimental conditions. Jour. Exp. Zool. **38**: 213–244.

MORGAN, T. H., 1914 The failure of ether to produce mutations in Drosophila. Amer. Nat. **48**: 705–711.

MULLER, H. J., 1917 An Oenothera-like case in Drosophila. Proc. Nat. Acad. Sci. **3**: 619–626.
1918 Genetic variability, twin hybrids, and constant hybrids, in a case of balanced lethal factors. Genetics **3**: 422–499.
1920a A quantitative study of mutation in the second chromosome of Drosophila. (Address, unpublished, to the Amer. Soc. of Nat., 38th annual meeting, in Chicago, Dec. 31, 1920.) Rec. Amer. Soc. Nat. **3**: 69.
1920b Further changes in the white eye series of Drosophila and their bearing on the manner of occurrence of mutation. Jour. Exp. Zool. **31**: 433–473.
1922 Variation due to change in the individual gene. Amer. Nat. **56**: 32–50.
1923a The measurement of mutation frequency made practicable. (Abstr.) Anat. Rec. **24**: 419.
1923b Recurrent mutations of normal genes of Drosophila not caused by crossing over. (Abstr.) Anat. Rec. **26**: 397–398.
1923c Mutation. Eugenics, Genetics, and the Family **1**: 106–112. (Read at Internat. Eug. Cong., New York City, Sept., 1921).
1926 The gene as the basis of life. (Address in symposium on "The Gene," at Internat. Cong. of Plant Sci., Ithaca, New York, Aug., 1926.) In press in Proc. of the Cong.
1927a Quantitative methods in genetic research. Amer. Nat. **61**: 407–419.

1927b Artificial transmutation of the gene. Science, N. S., **66**: 84–87.

1928 The problem of genic modification. (Address at Internat. Gen. Cong., Berlin, Sept., 1927.) Zeitschr indukt Abstamm. u. vererb. Sup.-Bd. **1**: 234-260.

MULLER, H. J., AND ALTENBURG, E., 1919 The rate of change of hereditary factors in Drosophila. Proc. Soc. Exp. Biol. Med. **17**: 10–14.

1921 A study of the character and mode of origin of eighteen mutations in the X-chromosome of Drosophila. (Abstr.) Anat. Record **20**: 213.

MULLER, H. J., and SETTLES, F., 1926 The non-functioning of the genes in spermatozoa. Zeitschr. indukt. Abstamm. u. Vererb. **43**: 285–312.

STURTEVANT, A. H., 1925 The effects of unequal crossing over at the bar locus in Drosophila. Genetics **10**: 117–147.

WARD, L., 1923 The genetics of curly wing in Drosophila. Another case of balanced lethal factors. Genetics **8**: 276–300.

ZELENY, G., 1921 The direction and frequency of mutation in the bar-eye series of multiple allelomorphs of Drosophila. Jour. Exp. Zool. **34**: 203–233.